TESIS DOCTORAL

Papel de los factores ambientales tempranos en la inmunocompetencia y el proceso de colonización intestinal en lactantes con riesgo de enfermedad celíaca. Inmunomodulación de potenciales probióticos.

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ABREVIATURAS

AEM	Antiendomisio
APC	Célula presentadora de antígeno (*Antigen presenting cell*)
AR	Artritis reumatoide
ATGt	Antitransglutaminasa tisular
CDmo	Células dendríticas derivadas de monocitos
CDs	Células dendríticas
CMNs	Células mononucleares de sangre periférica
DLG	Dieta libre de gluten
DT1	Diabetes tipo I
EC	Enfermedad celíaca
GWAS	Estudios de asociación del genoma completo (*Genome-wide association studies*)
HLA	Antígeno leucocitario humano (*Human leukocyte antigen*)
HMOs	Oligosacáridos de la leche humana (*Human milk oligosaccharides*)
IFN-γ	Interferón-gamma
Ig	Inmunoglobulina
IL	Interleuquina
LGG	*Lactobacillus rhamnosus* GG
LIEs	Linfocitos intraepiteliales intestinales
NK	*Natural killer*
PCR	Reacción en cadena de la polimerasa (*Polimerase chain reaction*)
SI	Sistema inmune
TGF-β	Factor de crecimiento transformante-beta (*Transforming growth factor-beta*)
TGt	Transglutaminasa tisular
TLR	Receptores tipo Toll (*Toll-like receptors*)
TNF-α	Factor de necrosis tumoral-alpha (*Tumor necrosis factor-alpha*)
T-RFLP	Polimorfismos en longitude de fragmentos de restricción terminales (*Terminal Restriction Fragment Length Polymorphism*)

RESUMEN GENERAL

El desarrollo del sistema inmune (SI) comienza antes del nacimiento y no termina hasta la adolescencia. Un correcto proceso de maduración desde los primeros días de vida es esencial para desarrollar respuestas inmunológicas correctas frente a posibles infecciones, y posiblemente evitar el desarrollo de alergias y enfermedades autoinmunes. El periodo postnatal inmediato al nacimiento está caracterizado por la permeabilidad intestinal a macromoléculas intactas, por lo que el desarrollo de una inmunidad de la mucosa efectiva es esencial para la protección frente a infecciones y el control de la exposición a alérgenos en el periodo postnatal. El SI de la mucosa es rápidamente estimulado por la colonización bacteriana y por superficies de cuerpos externos; esta interacción contribuye al completo desarrollo de la microbiota intestinal que es, a su vez, esencial para un buen desarrollo del SI sistémico. Existen diversos factores ambientales que pueden alterar el desarrollo del SI y provocar enfermedades inmunológicas como alergias, asma, diabetes tipo I, o enfermedad celíaca (EC). Pero a la vez, existen estudios preliminares que demuestran la capacidad inmunomoduladora de los probióticos y su posible utilidad en la prevención de alguna de estas enfermedades.

El presente trabajo forma parte del estudio PROFICEL el cual va dirigido a identificar qué factores ambientales pueden estar implicados en el desarrollo de EC en individuos con susceptibilidad genética, y su posible prevención a través de potenciales probióticos.

Por todo ello, el objetivo de este trabajo ha sido estudiar el efecto conjunto del genotipo HLA-DQ y de diversos factores ambientales sobre el desarrollo del SI y el establecimiento de la microbiota intestinal de niños con riesgo familiar de EC, para en un futuro identificar los principales factores que contribuyen a la susceptibilidad de padecer EC. Un segundo objetivo ha sido evaluar *in vitro* la capacidad de inmunomodulación de diferentes cepas bacterianas características de niños alimentados con leche materna y leche de fórmula, para estudiar posibles efectos beneficiosos de la leche materna sobre la EC e identificar posibles cepas bacterianas probióticas.

En primer lugar estudiamos la influencia de la lactancia materna *vs.* fórmula y su interacción con el genotipo HLA-DQ sobre las poblaciones linfocitarias de niños con riesgo genético de desarrollar EC. No se encontró ninguna interacción entre el tipo de lactancia y el genotipo de riesgo HLA-DQ. Por el contrario sí encontramos efecto del tipo de lactancia sobre las subpoblaciones de linfocitos. En niños alimentados con leche de materna el número absoluto de células CD4+CD38+ fue menor y el porcentaje de células CD4+CD25+ fue mayor que en los niños alimentados con leche de fórmula. Estos resultados sugieren que los niños alimentados con leche materna podrían tener un sistema inmune más maduro que los niños alimentados con leche de

fórmula, y que el efecto de la lactancia materna sobre las subpoblaciones de linfocitos podría ser beneficioso en niños con riesgo de desarrollar EC.

En segundo lugar estudiamos la influencia de diversos factores ambientales sobre las poblaciones linfocitarias y la microbiota intestinal de niños con riesgo a desarrollar EC. La lactancia de fórmula se asoció con un mayor número absoluto de linfocitos, células CD3+, CD4+, CD4+CD38+, CD4+CD28+ y CD3+CD4+CD45RO+. La infecciones sufridas durante los primeros meses de vida se asociaron con un aumento de los linfocitos T colaboradores (CD4+), particularmente, los linfocitos T maduros (CD45RO+) y activados (CD25+ y HLA-DR+). El parto por cesárea y la administración de la vacuna de rotavirus se asociaron a un menor porcentaje de células CD4+CD25+. Referente a la composición de la microbiota intestinal, la toma temprana de antibióticos se asoció y correlacionó con un menor número de *Bifidobacterium longum* y un mayor número de *Bacteroides fragilis.* Según nuestros resultados parece ser que las infecciones y la toma de antibióticos en los primeros 4 meses de edad son los factores ambientales tempranos más fuertemente y/o frecuentemente asociados a las subpoblaciones de linfocitos y la composición de la microbiota, respectivamente, en niños con riesgo de EC.

Por último, estudiamos el efecto inmunomodulador de diferentes cepas bacterianas características de niños alimentados con leche materna y leche de fórmula mediante cultivo directo con células mononucleares de sangre periférica (CMNs), y co-cultivo con CMNs y células Caco-2. *Bifidobacterium catenulatum* y *Bifidobacterium breve* fueron las más fuertes inductoras de la producción de IFN-γ por estimulación directa de CMNs. *B. longum* fue la mayor inductora de IL-10 y la menor de TNF-α. En el sistema de co-cultivo CMNs/Caco-2, *B.breve* fue la mayor inductora de IL-8 por parte de las células Caco-2, y significativamente diferente de *B. infantis*, *B. adolescentis*, y la mezcla bacteriana característica de niños alimentados con lactancia de fórmula (LF) ($P<0.05$). El IFN-γ producido por las CMNs estimuladas con la mezcla bacteriana característica de niños alimentados con lactancia materna (LM) (que contiene 22% de *B. breve*, comparado con un 7% en la mezcla LF) fue significativamente mayor comparado con *B. adolescentis*, *B. infantis* y *B. longum*. También, *B. adolescentis* inhibió la producción de IFN-γ comparado con la mezcla LF y *B. longum*. De estos resultados se puede concluir que la proporción de las diferentes cepas de bifidobacterias parece ser un importante determinante del balance de citoquinas en el ambiente intestinal simulado en el estudio. *B. breve* y la combinación de las especies de *Bifidobacterium* típicamente encontradas en la microbiota de niños alimentados con leche materna mostraron los efectos inmunoestimuladores más significativos.

Los resultados obtenidos en este trabajo permiten avanzar en el conocimiento del papel de factores ambientales tempranos sobre el desarrollo del SI y la microbiota en niños con riesgo de

EC, y proporcionan evidencias sobre las características inmunomoduladoras de posibles cepas probióticas dirigidas a la prevención de enfermedades asociadas al SI, como puede ser la EC.

INTRODUCCIÓN

1. DESARROLLO DEL SISTEMA INMUNE

El sistema inmune (SI) humano comienza a desarrollarse en el feto. El SI del feto está polarizado hacia una respuesta Th2, para evitar abortos espontáneos debidos a las citoquinas pro-inflamatorias (respuesta Th1). Durante el periodo neonatal e infantil sufre un constante proceso de maduración gracias a la exposición a productos ambientales microbianos que activan y aceleran el proceso, disminuyendo la polarización celular hacia una respuesta Th2 y/o aumentando la polarización hacia una respuesta Th1. Un desarrollo tanto acelerado como retardado puede dar lugar a un sistema inmunológico defectuoso [1], por lo que un correcto proceso de maduración desde los primeros días de vida es esencial para desarrollar respuestas inmunológicas correctas frente a posibles infecciones, y posiblemente evitar el desarrollo de alergias y enfermedades autoinmunes.

1.1. Inmunidad innata

Las partes del SI responsables de los mecanismos de defensa inmediata y/o que no requieren exposición previa a un patógeno específico tradicionalmente hacen referencia a la inmunidad innata. Se caracteriza por la rápida eliminación de patógenos mediante la acción de los componentes del complemento, anticuerpos y lisis de las células infectadas del huésped, así como la fagocitosis llevada a cabo por granulocitos, monocitos, macrófagos y células dendríticas (CDs) [2, 3].

Los niveles de las proteínas solubles en plasma que participan en la inmunidad innata son menores en recién nacidos que en adultos. Las concentraciones en plasma de los componentes del complemento están disminuidas con respecto a adultos (10-70% de los niveles de adulto) [4]. Esta deficiencia puede dar lugar a la incapacidad de los recién nacidos de limitar la replicación de muchas cepas bacterianas en la sangre, y además como el complemento también participa en la inmunidad adaptativa, puede estar contribuyendo a la discapacidad del neonato en la respuesta adaptativa [5].

El estado de hipoxia del recién nacido justo después del parto aumenta la producción de las citoquinas interleuquina (IL)-6 e IL-8, las cuales pueden disparar respuestas de fase aguda. Se especula que la rápida activación de la respuesta de fase aguda justo después del nacimiento debe de estar dirigida hacia la eliminación de algunos productos microbianos que pueden haber translocado a través de las membranas mucosas y/o colonizaciones iniciales para evitar el exceso de inflamación cuando el recién nacido se pone en contacto con el mundo exterior [5]. La hipoxia también aumenta los niveles de adenosina, la cual posee propiedades inmunomoduladoras, e

inhibe la producción de la citoquina pro-inflamatoria factor de necrosis tumoral-alpha (TNF-α) en respuesta a lipopéptidos bacterianos [1].

Esta capacidad de producción de citoquinas en respuesta a estímulos infecciosos permanece baja durante años. Los monocitos de niños adquieren la capacidad de producir TNF-α e IL-6 a los mismos niveles de adulto alrededor de los 3 años, mientras que otras citoquinas como interferón-gamma (IFN-γ) e IL-12 permanecen bajas hasta la adolescencia [6].

Los recién nacidos tienen un número reducido de células progenitoras de granulocitos y monocitos inactivas, que da lugar a un menor número de neutrófilos durante condiciones de estrés (por ejemplo, en sepsis). Además los neutrófilos muestran discapacidad en múltiples aspectos funcionales, como el proceso de extravasación o diapédesis (quimiotaxis, adhesión, migración), y formación de lamelipodios [5]. Estos defectos en los neutrófilos son aun más pronunciados en bebés prematuros, pero comienzan a corregirse en las primeras semanas de vida [1, 5].

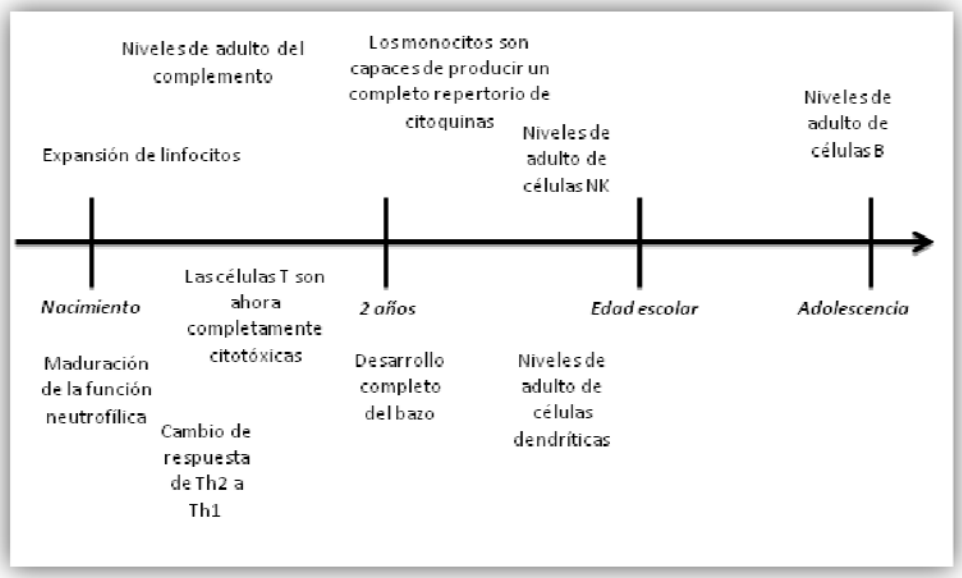

Figura 1. Desarrollo postnatal del sistema inmune (figura adaptada de *Ygberg S. and Nilsson A, 2012* [1])

Las CDs al nacimiento producen menos IL-12, la cual es la citoquina central de las CDs. La IL-12 puede considerarse como la citoquina de unión entre la inmunidad innata y la activación de la inmunidad adaptativa, de ahí que la inmunidad adaptativa esté discapacitada en las etapas tempranas de la vida. Los niveles de CDs aumentan con la edad, llegando a doblarse alrededor de los 5 años [1].

El número absoluto de células *natural killer* (NK) alcanza sus niveles máximos en el nacimiento. Después bajan progresivamente hasta alcanzar los niveles del adulto alrededor de los 5 años. Aunque el número de células NK sea alto en niños pequeños, la función citotóxica está reducida [1]. Esto puede ser debido a los bajos niveles de citoquinas activadoras, ya que la citotoxicidad puede ser restaurada *in vitro* añadiendo citoquinas [7].

1.2. Inmunidad adaptativa

En contraste con la inmunidad innata, la adaptativa está dirigida a eliminar patógenos de forma específica, y a crear la memoria inmunológica. Esto se lleva a cabo gracias a varios tipos de células T y células B [3, 8]. Durante las primeras semanas de vida, el neonato experimenta un incremento masivo de linfocitos, que es independiente de la edad gestacional, por lo que se piensa que el desarrollo de los linfocitos está unido a la vida extrauterina [1].

Los análisis de células sanguíneas muestran una marcada linfocitosis al nacimiento y en etapas posteriores comparado con la edad adulta. Durante las primeras semanas de vida se produce un incremento de linfocitos T y B mientras que las células NK disminuyen bruscamente inmediatamente después del nacimiento [9, 10].

Los niveles de linfocitos T son altos al nacimiento y se incrementan durante los dos primeros años de vida, para luego decrecer y llegar a los niveles normales de la edad adulta en edad escolar [10, 11]. Los linfocitos T colaboradores (CD3+CD4+) muestran el mismo patrón, pero los linfocitos T citotóxicos (CD3+CD8+) permanecen estables hasta los 2 años y después decrecen gradualmente hasta los niveles del adulto [11]. Hasta los 2 años de edad los niños presentan un alto ratio células CD4+/células CD8+, y esto es debido principalmente al gran pool de células T colaboradoras naíf (CD3+CD4+CD45RA+) en expansión presentes durante el primer año de vida [12, 13]. Los linfocitos T colaboradores de memoria (CD3+CD4+CDRO+) están casi ausentes en el nacimiento y se incrementan gradualmente en los años posteriores [11]. Los linfocitos T citotóxicos de memoria (CD3+CD8+CDRO+) están ya presentes en el nacimiento y muestran una expansión hasta la edad adulta [11]. El número absoluto de las células T reguladoras (CD3+CD4+CD25+CD127-) muestran un patrón parecido a los linfocitos T colaboradores naíf, aumentan durante el primer año de vida y después decrecen hasta los niveles de la edad adulta [11]. Las proporciones de las células T activadas como CD3+CD25+ y CD3+HLA-DR+ son más bajas en recién nacidos que en adultos [14]. Por el contrario, las proporciones de células CD8+CD28+, CD8+CD38+, y CD4+CD38+ son mayores en neonatos [14, 15].

El número de linfocitos B se duplica durante los primeros meses de vida y después decrece casi cinco veces, gradualmente, desde la segunda mitad del primer año de vida hasta la edad adulta [16]. Esto es causado casi totalmente por la expansión del pool de linfocitos B naíf, que son más altos en los grupos de edad más jóvenes [16].

Tabla 1. Números absolutos de las subpoblaciones de linfocitos en sangre periférica (*Schatorjé EJH, 2011* [11])

	Grupos de edad									
Subpoblaciones de linfocitos	Cordón umbilical (N=18)	1 s-2 m (N=11)	2-5 m (N=12)	5-9 m (N=13)	9-15 m (N=10)	15-24 m (N=10)	2-5 a (N=11)	5-10 a (N=15)	10-16 a (N=15)	Adultos (N=21)
Linfocitos totales	5.4 (3.1–9.4)	5.7 (2.9–11.4)	6.5 (3.4–12.2)	5.8 (1.8–18.7)	6.3 (3.2–12.3)	4.1 (1.4–12.1)	2.7 (1.4–5.5)	2.4 (1.2–4.7)	2.4 (1.4–4.2)	2.3 (1.2–4.1)
Linfocitos B	0.54 (0.14–2.0)	0.81 (0.18–3.5)	1.1 (0.52–2.3)	0.90 (0.13–6.3)	0.94 (0.1–7.7)	0.76 (0.2–3.7)	0.49 (0.2–1.3)	0.29 (0.1–0.8)	0.30 (0.1–0.7)	0.23 (0.06–0.8)
Células NK	1.2 (0.5–3.1)	0.51 (0.14–1.9)	0.44 (0.10–2.0)	0.52 (0.07–3.9)	0.50 (0.07–3.5)	0.47 (0.055–4.0)	0.18 (0.1–0.5)	0.20 (0.07–0.6)	0.33 (0.09–1.2)	0.34 (0.10–1.2)
Linfocitos T (CD3+)	3.1 (1.4–6.8)	4.0 (1.9–8.4)	4.5 (2.2–9.2)	4.0 (1.4–11.5)	4.4 (2.4–8.3)	2.5 (0.7–8.8)	1.9 (0.8–4.3)	1.8 (0.8–4.0)	1.6 (0.85–3.2)	1.5 (0.7–3.0)
Linfocitos T colaboradores (CD3+CD4+)	2.2 (1.0–4.8)	3.0 (1.5–6.0)	3.3 (1.6–6.5)	2.7 (1.0–7.2)	3.0 (1.3–7.1)	1.6 (0.4–7.2)	1.1 (0.5–2.7)	1.0 (0.4–2.5)	0.9 (0.4–2.1)	1.0 (0.5–2.0)
Linfocitos T citotóxicos (CD3+CD8+)	0.8 (0.2–2.7)	0.9 (0.3–2.7)	1.0 (0.3–3.4)	1.1 (0.2–5.4)	1.2 (0.4–4.1)	0.7 (0.2–2.8)	0.6 (0.2–1.8)	0.6 (0.2–1.7)	0.6 (0.3–1.3)	0.5 (0.2–1.2)

S= semana; m=meses; a=años

Los datos son medias y intervalos de tolerancia del 90% x 10^9/L

1.3. Desarrollo de la inmunidad de la mucosa

En humanos, los componentes sistémicos y de la mucosa del SI adaptativo, aunque están interrelacionados, se desarrollan independientemente y a diferentes edades [17]. El SI de la mucosa en humanos comprende las estructuras linfoides asociadas a los tractos nasal, bronquial, gastrointestinal, y urogenital, así como las glándulas lacrimales, salivares y mamarias, y la membrana sinovial de las articulaciones [17]. Los tejidos linfoides asociados a la mucosa forman una red interconectada en la que sus receptores permiten la activación de las células plasmáticas en un lugar determinado, para luego ser trasladadas a otros lugares de la mucosa, proporcionando así una protección inmunológica extensiva a todas las superficies mucosas [18].

En ausencia de infecciones intrauterinas, el SI de la mucosa está desprovisto de linfocitos que expresan IgA, y no hay células B reactivas en los folículos linfoides intestinales al nacimiento [18]. Prenatalmente, la mayoría de las células de los centros germinales se marcan inmunohistoquímicamente para las inmunoglobulinas citoplasmáticas IgG o IgM, pero no IgA [19]. Este estado se invierte rápidamente después del parto en respuesta a la presentación antigénica. En el intestino el número de células que contienen IgM predominan hasta el primer mes de vida;

después, las células que contienen IgA predominan y continúan su incremento hasta los 2 años de edad [20].

El número de linfocitos intraepiteliales intestinales (LIEs) se expande después del nacimiento llegando a los niveles del adulto a los 2 años de edad. Esta expansión se debe principalmente al gran incremento de células TCRαβ+, aunque las células TCRγδ+ también se expanden pero en menor medida. Fenotípicamente, los LIEs son predominantemente células T citotóxicas (CD3+CD8+) desde el nacimiento hasta la edad adulta [21]. EL predominio de la subpoblación TCRαβ+CD8+ de los LIEs apoya el concepto de que estas células T están involucradas en la tolerancia oral a los antígenos de la dieta [17].

El SI de la mucosa es rápidamente estimulado por la colonización bacteriana y por superficies de cuerpos externos. Durante la colonización temprana en el intestino, los productos microbianos que se encuentran en el lumen se unen a los receptores tipo Toll (TLR) de las células epiteliales y de ese modo, pierden actividad, de otro modo crearían una respuesta inflamatoria dañina para el intestino [1]. El desarrollo de una inmunidad de la mucosa efectiva es esencial para la protección frente a infecciones y frente la exposición a alérgenos en el periodo postnatal [17]. El periodo postnatal inmediato al nacimiento está caracterizado por la permeabilidad intestinal a macromoléculas intactas. El rápido cierre de las membranas mucosas es un proceso importante ya que limita la exposición sistémica a antígenos, la cual si es tratada inapropiadamente, puede dar lugar a una fuerte infección, o a atopía, con la subsiguiente pérdida de tolerancia [22].

1.4. Influencia de la microbiota intestinal en el desarrollo del sistema inmune

El intestino humano contiene aproximadamente 10^{14} bacterias, las cuales constituyen la microbiota intestinal [23], que está compuesta principalmente por 5 filos bacterianos, *Firmicutes* (géneros *Clostridium, Staphylococcus, Lactobacillus*), *Bacteroidetes* (género *Bacteroides*), *Actinobacteria* (género *Bifidobacterium*), *Proteobacteria* (géneros *Escherichia, Klebsiella*, etc) *, y Fusobacteria* (género *Fusobacterium*) [24]. *Bacteroidetes* y *Firmicutes* son los filos intestinales más comunes, representando aproximadamente un 90% de la microbiota intestinal total [23, 24]. La microbiota intestinal se caracteriza por ser estable a lo largo de la vida, excepto en los periodos de establecimiento durante las primeras etapas de la vida (1-2 años) [25] y en la senectud [26], y algunos periodos en los que se dan situaciones patológicas o en los que tienen lugar factores de agresión al individuo (antibióticos, factores de estrés). La adquisición de la microbiota intestinal ocurre en el primer año de vida, y sigue unos pasos sucesivos, primero dominan los anaerobios facultativos como las enterobacterias, coliformes y lactobacilos, seguidos de los géneros anaerobios tales como *Bifidobacterium*, *Bacteroides* y *Clostridium*.

La composición de la microbiota intestinal depende de muchos factores, siendo el contacto físico con la madre, otros sujetos o elementos ambientales lo que más influye. Así, el contacto con la madre por medio del parto vaginal puede ser el primero de ellos. Incluso durante el embarazo, ya que se han encontrado bacterias de origen intestinal en el cordón umbilical y meconio de niños sanos [27, 28]. Otros factores conocidos con influencia en la microbiota son el tipo de lactancia (materna *vs*. fórmula), o el uso de antibióticos, entre otros [23, 24].

Gracias a los estudios realizados con animales libres de gérmenes (*germ-free*) comparados con animales colonizados convencionalmente, y los estudios realizados en humanos en los que se compara la microbiota de gente sana con la de pacientes que sufren enfermedades relacionadas con el SI, podemos conocer los efectos de la microbiota intestinal sobre el desarrollo del SI.

Los animales *germ-free* son animales mantenidos en condiciones de esterilidad, que no han tenido contacto alguno con microorganismos en ningún momento de su desarrollo prenatal y postnatal. En ratones *germ-free*, los niveles de inmunoglobulinas séricas se ven reducidos, el tamaño de sus bazos es menor, y el tejido linfoide asociado al intestino muestra un estado inmaduro, con un bajo contenido en la lámina propia de células T, y de células B productoras de inmunoglobulina (Ig) A, pero estas deficiencias son restauradas después de la colonización con bacterias comensales [29].

Los estudios con humanos también muestran que la colonización bacteriana del intestino del recién nacido modifica la producción de la IgA secretora salival, el número de células productoras de IgA y IgM en circulación, y la expresión de los receptores tipo Toll (TLRs) de la inmunidad innata en monocitos de sangre periférica [30, 31]. Un estudio prospectivo observacional también mostró que la alteración en la composición de la microbiota en etapas tempranas de la vida, caracterizada por un ratio reducido del número de *Bifidobacterium/Clostridium*, precede al desarrollo de dermatitis atópica [32], sugiriendo que la microbiota participa en el riesgo de desarrollo de enfermedades inmunológicas. En este contexto, Grönlund *y col.* [33] también han descrito diferencias entre la microbiota de las leches maternas de madres sanas y alérgicas, caracterizadas éstas últimas por una menor concentración de *Bifidobacterium* spp., así como el efecto de estas diferencias sobre los niveles de *Bifidobacterium* spp. en las heces de los lactantes.

2. INFLUENCIA DE LOS FACTORES AMBIENTALES EN EL DESARROLLO DEL SISTEMA INMUNE

El efecto de los factores ambientales sobre el desarrollo del SI es relevante en las primeras etapas de la vida, cuando en el intestino inmaduro del neonato tiene lugar el establecimiento de la microbiota y el SI adquiere una completa competencia y tolerancia hacia antígenos no dañinos [34].

Los factores ambientales que pueden influir sobre el SI, pueden ejercer su efecto durante el embarazo (prenatales), durante o entorno al parto (perinatales), y después del nacimiento (postnatales).

Nuevos estudios muestran que los factores ambientales y el estrés durante el parto pueden alterar el perfil inmune en el niño, e influir en el riesgo de enfermedades asociadas con el SI como el asma y la alergia. Los estudios más recientes se centran sobre el "imprinting" que ejercen los factores ambientales sobre la microbiota intestinal infantil, la cual es determinante en el desarrollo del SI. Los factores ambientales tempranos que impactan sobre la microbiota incluyen el tipo de parto, la dieta infantil, y el uso de antibióticos, factores que se han asociado también al asma y a las enfermedades alérgicas.

A continuación se describen estudios sobre el efecto de diferentes factores ambientales pre-, peri-, y postnatales sobre el SI y la microbiota.

2.1. Lactancia materna

La leche materna es el primer alimento al que se ve expuesto el recién nacido, y satisface todos sus requerimiento nutricionales y de desarrollo saludable, protegiendo a los neonatos de las infecciones. Este efecto protector se debe a los componentes presentes en la leche materna y el calostro, que incluyen carbohidratos, nucleótidos, ácidos grasos, inmunoglobulinas, citoquinas, células inmunocompetentes, lisozima, lactoferrina, lactadherina, y otras glicoproteínas y péptidos antimicrobianos [23, 35].

La leche materna también contiene factores que pueden modular las señales de los TLR, como el TLR2 soluble, el cual puede inhibir competitivamente la señal a través del receptor de membrana TLR2 [36]. De acuerdo a esto, se ha especulado que la reducción de la reactividad de el TLR2 en el nacimiento puede facilitar el establecimiento normal de bifidobacterias Gram-positivas beneficiosas de la flora intestinal [5].

La leche materna proporciona al neonato parte de la llamada "inmunidad adquirida a través de la madre", la cual es esencial en el periodo neonatal hasta que se desarrolla la inmunidad endógena. Fitzsimmons *y col.* [37] mostraron un aumento más rápido de la IgA total salival

durante los primeros 6 meses de vida en niños alimentados con leche materna comparado con los alimentados con leche de fórmula, y concluyeron que aunque la inmunidad secretora es inmadura en lactantes, la leche materna puede ayudar en la protección frente a microorganismos patógenos incrementando la velocidad de la maduración de la IgA de la mucosa. La leche materna también ha mostrado mejores respuestas a las vacunas [38].

Los oligosacáridos de la leche humana (HMOs) contribuyen a modular la composición de la microbiota y estimulan selectivamente el crecimiento de grupos bacterianos específicos, como bifidobacterias, en el intestino del lactante [39]. Así, en niños alimentados con leche materna predominan las especies de *Bifidobacterium*, representando más de un 90% de la microbiota fecal, mientras que en niños alimentados con leche de fórmula la microbiota es más heterogénea e integra miembros de géneros más diversos (*Streptococcus*, *Bacteroides*, *Clostridium*, *Bifidobacterium, etc*) [40, 41]. En los últimos años, se han detectado bacterias comensales en la leche materna de mujeres sanas [33, 42, 43]. Los principales géneros bacterianos que se han detectado en la leche materna son *Streptococcus* and *Staphylococcus*, representando el 36-65% y 29-50% del total de bacterias, respectivamente, mientras que el género *Bifidobacterium* sólo representa el 3-4% [44]. Aun así, las especies de *Bifidobacterium* llegan a ser predominantes en el intestino de los niños alimentados con leche materna probablemente debido a su mejor adaptación metabólica para utilizar los HMOs como se evidencia por su secuenciación genómica [45].

El mecanismo involucrado en la colonización bacteriana de la leche humana no está claro. Martín y col. (2003) [46] observaron diferentes bacterias ácido-lácticas en la piel de la mama y la leche materna sugiriendo que estas bacterias tenían orígenes diferentes y propusieron una vía endógena entero-mamaria para la colonización de la leche materna. Se ha demostrado que las CDs pueden atravesar el epitelio intestinal y capturar bacterias del lumen intestinal [47], y que además dichas bacterias se conservan viables durante días [48]. Martín y col. [49] han sugerido que, dado que hay una circulación de células del SI dentro del sistema linfoide asociado a mucosas, las bacterias podrían transportarse dentro de la célula dendrítica a otras localizaciones, incluida la glándula mamaria.

2.2. Tipo de parto y antibióticos

Existen estudios que han demostrado la influencia del tipo de parto (cesárea *vs.* vaginal) y la exposición a antibióticos sobre el establecimiento de la microbiota intestinal en las primeras etapas de la vida. Como anteriormente se ha descrito, un buen establecimiento de la microbiota intestinal es necesario para un desarrollo saludable del SI, por tanto, el tipo de parto y la exposición a antibióticos puede influir también en el posible desarrollo de enfermedades asociadas al SI. A continuación, se describen algunos estudios que demuestran las afirmaciones anteriores.

Biasucci *y col.* [50] evaluaron la relación entre el ecosistema intestinal del neonato y el tipo de parto (vaginal *vs.* cesárea). Para ello analizaron mediante la técnica de reacción en cadena de la polimerasa (PCR), con amplificaciones específicas para 10 especies diferentes de *Bifidobacterium,* 3 especies de *Ruminococcus* y *Bacteroides,* la microbiota de 23 niños nacidos por cesárea y 23 niños nacidos por parto vaginal a los 3 días de vida. La microbiota intestinal de los niños nacidos por cesárea era menos diversa, en términos de especies bacterianas, que la de los niños nacidos vaginalmente. Además, la microbiota intestinal después del parto por cesárea estaba caracterizada por la ausencia de especies del grupo *Bifidobacterium*, y los niños nacidos vaginalmente se caracterizaron por el predominio de bifidobacterias, como *B. longum* y *B. catenulatum*. Otro estudio analizó la influencia de la exposición a antibióticos y el tipo de parto en el periodo postnatal temprano sobre el desarrollo de la microbiota intestinal en 26 niños [51]. Dieciocho de ellos nacidos vaginalmente y sin exposición a antibióticos fueron usados como controles. Cinco niños fueron expuestos oralmente al antibiótico cefalexina en los 4 primeros días de vida, y 3 niños nacieron por cesárea. El análisis de la microbiota se llevó a cabo mediante polimorfismos de longitud de fragmentos de restricción terminales (T-RFLP) y PCR cuantitativa. Los T-RFLP mostraron menor diversidad en los niños tratados con antibióticos, especialmente en el género *Bifidobacterium*, comparado con el grupo control. La PCR cuantitativa mostró sobrecrecimiento de *Enterococcus* y un menor crecimiento de *Bifidobacterium*. Los niños nacidos por cesárea tuvieron una alteración muy similar a los niños tratados con antibióticos. Hill *y col*. [52], en un estudio con ratones, desarrollaron un protocolo de tratamiento oral con antibióticos y caracterizaron sus efectos en las comunidades bacterianas del intestino de ratón y la homeostasis de las células inmunes. Usando el método de "código de barras de ADN" (*DNA bar-coding*) y pirosecuenciación de segmentos del gen 16S del ADN ribosomal, examinaron los efectos temporales de los antibióticos en las comunidades bacterianas y encontraron reducciones significativas en la frecuencia de bacterias pertenecientes al filo *Firmicutes,* y en la persistencia de los filos *Bacteroidetes* y *Proteobacteria* conforme avanza el tiempo.

Adicionalmente, desarrollaron un análisis espacial de las comunidades bacterianas asociadas al lumen y la mucosa después del tratamiento con antibióticos y encontraron efectos significativos en el ciego, colon proximal, y colon distal, incluyendo la reducción en las especies asociadas a la mucosa del género *Lactobacillus*. Finalmente, examinaron los efectos del tratamiento oral con antibióticos en la homeostasis de las células inmunes de la mucosa y encontraron que la administración de antibióticos daba lugar a la reducción de la producción de RELM-β, una proteína secretada por las células caliciformes en respuesta a la colonización intestinal, así como la reducción de la producción de IFN-γ e IL-17 por los linfocitos T CD4+ de la mucosa.

Los estudios anteriores muestran la influencia del tipo de parto y el tratamiento con antibióticos sobre la microbiota intestinal. Los siguientes estudios que se describen muestran la influencia de estos factores ambientales en el desarrollo de enfermedades asociadas al SI.

Examinar si el parto por cesárea estaba asociado con el subsiguiente desarrollo de alergias alimentarias, fue el objetivo de Eggesbo *y col.* [53]. En una población de 2803 niños se obtuvo información acerca del tipo de parto, y el uso de antibióticos por parte de la madre y el niño. En los niños cuyas madres eran alérgicas, el parto por cesárea estaba asociado a un riesgo 4 veces mayor de alergia alimentaria al huevo. En los niños cuyas madres no eran alérgicas la asociación fue más débil y no significativa. Por tanto, el estudio indica que en niños predispuestos el parto por cesárea puede aumentar el riesgo de desarrollar alergias alimentarias. Renz-Polster [54] *y col.* llevaron a cabo un estudio retrospectivo en 8953 niños entre los 3 y 10 años de edad para determinar si los niños nacidos por cesárea tendrían un riesgo diferente de desarrollar enfermedades alérgicas que los niños nacidos por parto vaginal. Encontraron que el riesgo de ser diagnosticado con rinoconjuntivitis alérgica fue significativamente mayor en los niños nacidos por cesárea, y que el parto por cesárea estaba también asociado al subsiguiente diagnóstico de asma, pero no encontraron asociación entre el tipo de parto y dermatitis atópica. Según este estudio, el parto por cesárea está asociado a un incremento del riesgo de desarrollar rinoconjuntivitis alérgica y asma. En el estudio de cohorte desde el nacimiento “KOALA” [55], evaluaron la exposición a antibióticos en edad temprana y el posterior desarrollo de eczema, y sibilancia, y sensibilización alérgica en la infancia. Se recogió información sobre el uso de antibióticos durante los primeros 6 meses, y de desarrollo de eczema y sibilancia durante los 2 primeros años. La sensibilización alérgica se determinó a los 2 años de edad mediante análisis de inmunoglobulinas totales e inmunoglobulina E en muestras de sangre. Como resultados obtuvieron que el riesgo de sibilancia recurrente y prolongada fue mayor en niños expuestos directamente a antibióticos mediante medicación, y también la exposición a antibióticos a través de la lactancia materna se asoció con sibilancia recurrente, pero no con prolongada.

El desarrollo de eczema y sensibilización no mostraron asociación con la exposición a antibióticos. Por último, en el estudio prospectivo de cohorte desde el nacimiento de Dom y col. [56] también investigaron la exposición a antibióticos, esta vez pre- y post-natal, y el subsiguiente desarrollo de eczema, sibilancia recurrente y sensibilización atópica en 773 niños a los 4 años de edad. La exposición prenatal a antibióticos se asoció con la prevalencia de eczema mientras que no se encontró asociación con sibilancia recurrente y sensibilización atópica. Tampoco se encontró asociación entre la exposición a antibióticos a través de la lactancia materna y el desarrollo de eczema, sibilancia, o sensibilización atópica. Por último, se encontró asociación negativa entre el uso de antibióticos durante el primer año de vida y el desarrollo de eczema y sensibilización, y entre el uso de antibióticos después del primer año de vida y sibilancia recurrente, eczema y sensibilización. Por tanto, los autores concluyeron que la exposición indirecta a antibióticos incrementa el riesgo de síntomas alérgicos en niños, mientras que, al contrario que en el estudio "KOALA", la exposición directa parece ser protectora.

Parece ser que ciertos factores ambientales tales como el tipo de parto y la administración de antibióticos ejercen algún tipo de efecto sobre el desarrollo del SI, pero debido a la falta de concordancia de resultados entre los diversos estudios es necesario llevar acabo más investigaciones sobre el efecto de estos factores ambientales sobre el desarrollo del SI.

2.3. Infecciones

De acuerdo con la "*hipótesis de la higiene*" los cambios en el estilo de vida en ciudades industrializadas han dado lugar a una disminución del número de infecciones que se ha asociado a un aumento del número de enfermedades alérgicas y autoinmunes [57]. La hipótesis fue propuesta por primera vez por Strachan, quien observó una correlación inversa entre la fiebre del heno y el número de hermanos mayores [58]. La idea es que algunos agentes infecciosos son capaces de proteger contra un amplio espectro de enfermedades relacionadas con el SI.

A continuación se describen algunos estudios que sugieren efectos beneficiosos de agentes infecciosos sobre enfermedades inmunológicas.

2.3.1. Infecciones crónicas transmitidas oro-fecalmente

Los organismos transmitidos oro-fecalmente en los que se han centrado recientes estudios a favor de la hipótesis de la higiene incluyen *Helicobacter pylori*, *Salmonella*, virus de la hepatitis A, enterovirus, y *Toxoplasma gondii* [59]

Muchos helmintos son también trasmitidos oro-fecalmente o rápidamente contraídos del ambiente y deben ser considerados como parte de la microbiota.

2.3.2. Helmintos

En varios estudios se ha demostrado que la erradicación de helmintos incrementa la dermatitis atópica [60-62]. En un estudio prospectivo en Argentina, se observaron a 12 pacientes con esclerosis múltiple y con alta eosinofilia en sangre periférica asociada. Estos pacientes presentaban infecciones parasitarias y mostraron un menor número de exacerbaciones, y un incremento en la secreción de las citoquinas IL-10 y factor de crecimiento transformante-beta (TGF-β) por parte de sus células mononucleares de sangre periférica (CMNs) comparado con pacientes con esclerosis múltiple no infectados [63]. También se ha observado que la administración de huevos del parásito *Trichuris suis* en pacientes con enfermedad de Crohn [64] y colitis ulcerosa [65] mejoran significativamente los síntomas. Además en 2006, se publicó un meta-análisis de 33 estudios, que tuvo en cuenta algunos estudios discordantes, el cual encontró que el anquilostoma (*Necator americanus*) se asoció con una reducción de asma fuertemente significativa [66].

2.3.3. Infecciones víricas

La primera pregunta que surge acerca de las infecciones víricas es si son protectoras, o al contrario, son el origen de algunas enfermedades inmunológicas.

Cuando consideramos las infecciones en el contexto evolucionario, claramente entendemos que el papel protector de las infecciones víricas infantiles contemporáneas no encaja en este contexto, ya que no han formado parte del ambiente en el que los humanos evolucionaron. Como se puede esperar, varios estudios concluyen que las infecciones padecidas durante la infancia no protegen frente a enfermedades alérgicas [67], diabetes tipo I (DT1) [68], o enfermedades inflamatorias intestinales [69, 70].

Sin embargo el momento en el que se padece la infección es crucial, ya que el coxsackievirus o el rotavirus parecen provocar autoinmunidad cuando se padecen tarde (por ejemplo, en el destete), pero pueden ser protectores cuando se padecen a edades muy tempranas [71, 72]. Esto sugiere otra cara de la hipótesis de la higiene, en la que la higiene moderna provocaría un retraso en la transmisión oro-fecal, por lo que las infecciones víricas se producen más tarde que en la evolución normal humana. Por lo tanto, las infecciones víricas serían protectoras durante los primeros meses de vida (durante la lactancia), pero no lo serían cuando se dan más tarde.

2.4. Dieta de la madre

La dieta de la madre durante el embarazo, también puede influir en el posterior desarrollo de alergias, eczema o asma en niños [73-76].

Un estudio del efecto de la adherencia de madres a la dieta Mediterránea sobre el desarrollo de asma o atopía en 460 niños de 6,5 años de edad, mostró una reducción del riesgo de sibilancia y atopía en niños cuyas madres tuvieron una alta adherencia a la dieta Mediterránea durante el embarazo [73]. Otros estudios con mayor tamaño muestral (2000-3000) basados en el estudio "*Finnish Type 1 Diabetes Prediction an Prevention Nutrition Study",* han estudiado el efecto de los antioxidantes, los ácidos grasos y la vitamina D de la dieta de la madre durante el embarazo sobre el desarrollo de alergias y asma en sus hijos. En una cohorte de 1669 niños de este estudio encontraron que una alta ingesta materna de Vitamina D durante el embarazo se asociaba a un bajo riesgo de desarrollo de enfermedades alérgicas en los niños a la edad de 5 años [74]. Otra cohorte de 2441 niños, mostró que un alto consumo materno de mantequilla o un alto ratio de ácidos grasos n-6:n-3 durante el embarazo estaba asociado a un incremento del riesgo de rinitis alérgica, y un alto consumo materno de ácidos grasos poliinsaturados totales y ácido graso α-linolénico se asoció a una disminución del riesgo de rinitis en los niños a la edad de 5 años [75]. Estos mismos autores estudiaron el efecto de la ingesta materna de antioxidantes, no encontrando ninguna asociación con el riesgo de asma, rinitis y eczema en sus hijos, aunque sí encontraron asociación entre la ingesta materna de magnesio y disminución del riesgo de eczema [76]. Aun así, los autores concluyen que se necesitan más evidencias para confirmar y definir más claramente el papel de cada uno de estos componentes de la dieta sobre el futuro desarrollo y alta prevalencia actual de enfermedades alérgicas.

2.5. Hipótesis de los viejos amigos

La "hipótesis de la higiene" ha sido reformulada recientemente y ha pasado a llamarse "hipótesis de los viejos amigos" (en inglés, *Old Friends Hypothesis*) [59]. Sugiere que desde el comienzo de la moderna urbanización a comienzos del siglo XIX ha habido un aumento progresivo de los problemas inmunorreguladores atribuibles a la desaparición de los organismos con los que co-evolucionaron los mamíferos en el entorno urbano ("viejos amigos"), y esto ha sido llevado a cabo por las fuerzas co-evolutivas con un papel crucial en el establecimiento de niveles "normales" de inmunorregulación. Esta hipótesis defiende que los factores más fuertemente implicados, según datos epidemiológicos, experimentales y de ensayos clínicos, son la microbiota

intestinal, los helmintos, y la transmisión fecal-oral de otros “viejos amigos” (Salmonella, virus de 29

la hepatitis A). Claramente la disminución de la exposición a helmintos, organismos fecales de otros humanos, y especies fermentadoras ambientales como los lactobacilos tendrá efectos directos sobre la composición de la microbiota. También los cambios producidos directamente sobre el SI por una disminución de la exposición a “viejos amigos” como helmintos, puede modular indirectamente la interacción huésped-microbiota [59]. Una vez que se producen problemas en el sistema de inmunorregulación, existen otros factores ambientales secundarios que empeoran la situación (Figura 2). El principal problema del aumento de enfermedades relacionadas con el SI es la influencia de los diversos factores en la inmunoregulación, y no es debido únicamente a la hipótesis de la higiene, o a la deficiencia de vitamina D, o a los virus, o a las dioxinas, o la dieta.

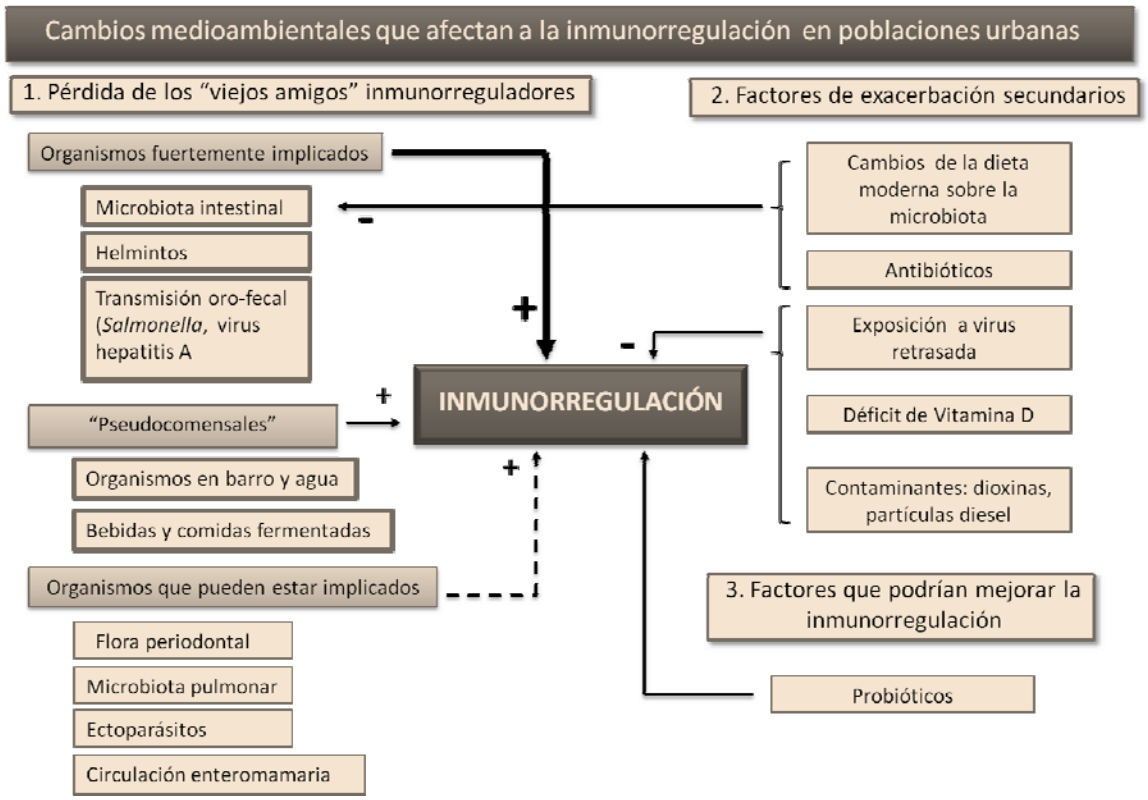

Figura 2. Factores que afectan a la inmunorregulación (figura adaptada de *Rook GA, 2012* [58])

2.6. Probióticos

Los probióticos son organismos vivos que cuando son administrados en una cantidad adecuada confieren al huésped un beneficio para la salud [77].

Está generalmente aceptado que la microbiota intestinal autóctona es capaz de modular las repuestas inmunológicas a través de la interacción con las células del SI en la mucosa intestinal, e influir en el desarrollo del SI de recién nacidos y niños, mientras que se cree que la alteración en la composición de la microbiota puede influir en la patogénesis de enfermedades alérgicas [78]. Existen estudios de suplementación con probióticos realizados a madres embarazadas y en periodo de lactancia, en los que se han obtenido resultados a favor de la capacidad de dichos probióticos de proteger frente a enfermedades asociadas al SI en los niños lactantes [79-81]. Una posible explicación podría ser el mecanismo de transmisión de las cepas probióticas entre madre e hijo, comentado anteriormente, propuesto por Martín *y col.* [49].

A continuación, se presentarán con mayor detalle y a modo de ejemplo estudios con probióticos tanto *in vitro*, como en humanos. Existe un gran número de trabajos publicados en torno a las propiedades inmunomoduladoras de los probióticos, por lo que sería complejo y excesivo pretender dar cuenta aquí de todos ellos. Sin embargo, cabe mencionar que gracias a la amplia atención que están recibiendo por parte de la comunidad científica, se están realizando avances que permiten delimitar el grado de evidencia científica con que cuenta una determinada cepa probiótica para su empleo con un fin protector relacionado con el mantenimiento de la salud, o la prevención o tratamiento de enfermedades.

3. PROBIÓTICOS

3.1. Papel inmunomodulador de los probióticos

Diversos estudios *in vitro* han evaluado el efecto de diferentes cepas bacterianas potencialmente probióticas en la modulación de la producción de citoquinas por células del epitelio intestinal, células dendríticas derivadas de monocitos (CDmo), y CMNs [82-85].

Se investigó el papel protector de las cepas probióticas *Lactobacillus acidophilus* Bar13, *L. plantarum* Bar10, *Bifidobacterium longum* Bar33 and *B. lactis* Bar30 frente a infecciones de enteropatógenos como *Salmonella typhimurium* y *Escherichia coli* H10407 en cultivos de células Caco-2 y HT-29 [82]. Estas cepas probióticas fueron efectivas en desplazar las especies patógenas, inhibiendo su adhesión a células Caco-2. Además, *L. acidophilus* Bar13 y *B. longum* Bar33 mostraron tener potencial para proteger a las células intestinales frente a una respuesta inflamatoria aguda. Ambas cepas probióticas inhibieron la producción de IL-8 por las células HT-29 estimuladas con TNF-α, IL-1β y LPS. De este estudio se concluye que las cepas probióticas *L. acidophilus* Bar13 y *B. longum* Bar33 son buenos candidatos para el desarrollo de alimentos funcionales útiles en la lucha contra las infecciones enteropatógenas. Se ha demostrado también que ciertas cepas probióticas como *Bifidobacterium breve* Bb99, *Streptococcus thermophilus* THS, y *Lactobacillus lactis* subsp. *cremoris* ARH74 tienen capacidad para aumentar la expresión de los marcadores de maduración de CDmo [83]. Sin embargo, difieren en la capacidad para inducir la expresión de citoquinas en las CDmo. *S. thermophilus* induce la expresión de citoquinas proinflamatorias (TNF-α, IL-12, IL-6, IFN-γ, and CCL20), mientras que *B. breve* y *L. lactis* son potentes inductores de IL-10. En otro estudio se utilizaron las cepas bacterianas *Bifidobacterium bifidum* W23*, Bifidobacterium breve* W6*, Bifidobacterium infantis* W52*, Bifidobacterium lactis* W18*, Bifidobacterium longum* W51*, Lactobacillus brevis* W63*, Lactobacillus casei* W56*, Lactobacillus Paracasei* W72*, Lactobacillus plantarum* W59*, Lactobacillus helveticus* W60*, Lactobacillus rhamnosus* W71*, Lactobacillus salivarius* W24*, y Lactococcus lactis* W58 para analizar su capacidad de modular *in vitro* la producción de citoquinas por CMNs, y seleccionar aquellas con un mayor potencial probiótico [84]. En este estudio, finalmente, se seleccionaron las cepas *B. bifidum*, *B. infantis* y *L. lactis* por su buena capacidad de inducir IL-10, e inhibir IL-5 e IL-3, para llevar a cabo un ensayo clínico de prevención de enfermedades alérgicas por bacterias probióticas. Elmadfa *y col*. [85] compararon los efectos inmunológicos de bacterias ácido-lácticas convencionales y probióticas en mujeres jóvenes y sanas, las cuales recibieron una bebida comercializada de leche fermentada con bacterias probióticas o

yogurt convencional durante 4 semanas. Después del tratamiento y de un periodo de lavado de 2 semanas se aisló la sangre de cada una de las voluntarias para analizar la citotoxicidad celular frente a células cancerosas, la activación de linfocitos T inducida por mitógenos, y la producción de citoquinas. Ambos tratamientos mostraron un mayor porcentaje de linfocitos T colaboradores CD69+ activados, y una mayor citotoxicidad después de su consumo, comparado con el punto basal. Además, la secreción de IL-10 se redujo significativamente después de la ingesta de la bebida probiótica y luego mostró un incremento marcado después del periodo de lavado, comparado con los niveles basales. Para estudiar los mecanismos existentes detrás de estos efectos se compararon dos cepas de bacterias ácido-lácticas, una convencional (*Lactobacillus delbrueckii*) y otra probiótica (*Lactobacillus rhamnosus* GG (LGG)) mediante un experimento *in vitro* de cultivo directo e indirecto (con una monocapa de células Caco-2), con CDs y CMNs por separado. LGG fue más potente en inducir la maduración de CDs, y *L. delbrueckii* produjo una mayor secreción de citoquinas proinflamatorias, así como IL-10. Estos efectos sólo se observaron en la incubación directa de bacterias y CDs o CMNs, no cuando ambas fueron separadas por una monocapa de células Caco-2, sugiriendo un papel modulador de las células del epitelio intestinal sobre las respuestas inmunes. A este respecto, se ha puesto de manifiesto el papel de las células del epitelio intestinal en el mantenimiento de la homeostasis inmunológica intestinal gracias a su papel como barrera física entre el contenido luminal y las células inmunes subyacentes, y también a las señales que envían a esas células inmunes desencadenadas por el contenido intestinal y la microbiota, incluyendo la secreción de defensinas, mucinas, quimioquinas y citoquinas [86].

3.2. Papel de los probióticos en la prevención de enfermedades relacionadas con el sistema inmune

Las bacterias presentes en la leche materna pueden jugar un papel clave en la reducción de la incidencia y gravedad de las infecciones del lactante, y se ha descrito que la administración de determinados probióticos a los lactantes supone una reducción de la incidencia de fenómenos alérgicos e inflamatorios [35].

En efecto, existen numerosos estudios en humanos que describen el efecto de determinadas cepas bacterianas probióticas sobre el estado de salud de niños lactantes. A continuación, se describen brevemente algunos de ellos.

Se evaluó el efecto de LGG en la prevención de enfermedad atópica en la descendencia de un grupo de mujeres embarazadas que pertenecían a familias que padecían esta enfermedad [79]. Las madres consumieron un suplemento que contenía dicha cepa probiótica durante 4 semanas al final

de su embarazo y durante el periodo de lactancia hasta los 3 meses de vida del bebé. De acuerdo a los resultados la concentración del TGF-β antiinflamatorio, aumentó la inmunoprotección proporcionada por la leche materna de las madres que recibieron probióticos en comparación con las que recibieron placebo. Además, en los niños cuyas madres recibieron probióticos se redujo significativamente el riesgo de desarrollar eczema atópico durante los primeros 2 años de vida comparado con el grupo placebo. Dichos resultados respaldan la capacidad de protección frente eczema atópico de la cepa LGG durante los dos primeros años de vida. Seguimientos de estos niños han sido publicados posteriormente a la edad de 4 y 7 años [87-89]. Se comprobó que en los tres periodos de seguimiento había una prevención significativa de entre 50 y 36% de los casos en el grupo suplementado con probióticos. Sin embargo, no se detectó ninguna diferencia entre ambos grupos en el porcentaje de niños con respuestas positivas al test cutáneo (SPT+) frente a una extensa batería de alérgenos, ni en el porcentaje de niños con elevada IgE total o específica de alérgeno en plasma. Quiere esto decir que no hay diferencias en la incidencia acumulada de eczema atópico, y que la prevención de eczema en general podría involucrar por ejemplo una protección de las barreras (como la piel y las mucosas), sin modificación de la participación de la IgE. En otro estudio [80] realizado en mujeres embarazadas de niños con un alto riesgo de enfermedad alérgica, se les dio a las madres cápsulas que contenían LGG, *Lactobacillus rhamnosus* LC705, *Bifidobacterium breve* Bb99 y *Propionibacterium fredenreichii spp. shermanii* JS durante 2-4 semanas antes del parto, y sus hijos recibieron la misma combinación de probióticos más galacto-oligosacáridos o placebo durante 6 meses. Se observó que en los niños con dos años de edad el tratamiento no había tenido ningún efecto sobre la incidencia acumulativa de enfermedades alérgicas, aunque sí fue capaz de disminuir significativamente la sensibilización a antígeno, el eczema y el eczema atópico, apoyando la asociación inversa entre eczema atópico y la colonización intestinal por probióticos. Se realizó un nuevo análisis cuando los niños habían cumplido 5 años [90], y encontraron prevención significativa de la incidencia de enfermedades atópicas sólo en los niños que habían nacido por cesárea. En otro estudio se comprobó el efecto de los probióticos indirectamente a través de la leche materna [81]. El objetivo fue determinar si la ingesta durante 6 meses de probióticos (*Lactobacillus casei*) sería capaz de modular el SI de las madres que habían dado a luz recientemente y estaban dando lactancia materna a sus bebés. Para ello se evaluaron las modificaciones de las citoquinas en la leche materna en tres etapas de la lactancia: el calostro, la leche temprana (10 días) y la leche madura (45 días). Además, se realizó un estudio de la antropometría, así como de episodios infecciosos y alérgicos justo después del nacimiento y durante un seguimiento hasta los 6 meses de vida. Se concluyó que la ingesta de leche fermentada con *L.casei* durante el periodo de lactancia contribuía modestamente a la modulación de la

respuesta inmune de la madre después del parto y disminuía la incidencia de episodios gastrointestinales en el niño alimentado con la leche materna procedente de las madres que habían consumido el probiótico. Como último ejemplo, Hoppu y col. [91] evaluaron los efectos de una intervención dietética (asesoramiento dietético y provisión de productos alimenticios derivados del aceite de colza) y administración de los probióticos LGG y *Bifidobacterium* lactis Bb12 en madres embarazadas sobre la composición de ácidos grasos y citoquinas en el calostro y la leche materna. Las proporciones de ácidos grasos n-3 totales, y las concentraciones de citoquinas TNF-α, IL-10, IL-4 e IL-2 fueron mayores en el grupo de intervención que en el grupo control. Además los ácidos grasos n-3 totales se asociaron positivamente con las citoquinas IFN-γ, TNF-α, IL-10, IL-4 e IL-2 en las muestras de calostro. Basándose en estudios previos en los que se han encontrado niveles más bajos de ácidos grasos n-3 y n-6 en el suero y leche materna de sujetos alérgicos, y en que las diferencias en la composición de ácidos grasos en la leche materna de madres atópicas y no atópicas pueden ser explicadas por la dieta, los autores sugieren la posibilidad de modificar los factores inmunomodulares de la leche materna mediante la dieta y la administración de probióticos.

Si se contemplan simultáneamente los múltiples estudios *in vivo* que han sido publicados en relación a la prevención de enfermedades tempranas, se observa que, cada cepa o combinación de ellas ejerce diferentes efectos sobre el SI. Como ejemplo, cabe mencionar el estudio de Wickens *y col.* [92], en el que encontraron que la suplementación con *Lactobacillus rhamnosus* HN001, pero no con *Bifidobacterium animalis* subsp. *lactis* HN019, redujo substancialmente la prevalencia de eczema a los dos años de edad. Aun así, los efectos moduladores de los probióticos durante la etapa perinatal parecen participar en la prevención de determinadas enfermedades del neonato, pero es necesario investigar más a fondo el papel de estas bacterias, por separado y en combinación, en el desarrollo del SI en las primeras etapas de la vida.

4. ENFERMEDAD CELÍACA

La enfermedad celíaca (EC) es una enteropatía con base inmunológica disparada por el gluten que contiene el trigo, la cebada y el centeno, cuando es consumido por individuos genéticamente susceptibles. Las características histológicas de la EC son la atrofia de los *villi* intestinales, la hiperplasia celular de las criptas intestinales, y el incremento en el número de linfocitos intraepiteliales. Está generalmente aceptado que la EC es una enfermedad mediada por células T, en la que los péptidos derivados de gliadina activan la infiltración de linfocitos T a la lámina propia, con la posterior liberación de citoquinas pro-inflamatorias, en particular IFN-γ. No obstante, la respuesta innata, con la participación de la IL-15, parece participar también de forma decisiva en el desarrollo de la enfermedad [93, 94]. Ambas citoquinas podrían participar en la activación de la citotoxicidad de los linfocitos intraepiteliales (LIEs) dando lugar a una profunda remodelación tisular. La EC es un trastorno complejo en cuya etiología participan factores ambientales y genéticos. El principal factor de riesgo genético en la EC está representado por los genes HLA-DQ pertenecientes al sistema de Antígenos humanos leucocitarios (HLA, del inglés, *Human Leukocyte Antigen*), que explican aproximadamente un 40% del riesgo genético de la enfermedad. Aproximadamente un 90-95% de los pacientes celíacos expresan los heterodímeros HLA-DQ2, y el 5-10% de pacientes restantes expresan el heterodímero HLA-DQ8 [94]. Pero todavía no está claro el por qué sólo un subgrupo de personas que expresan los heterodímeros HLA-DQ2 y HLA-DQ8 desarrolla la enfermedad, y de este subgrupo, por qué unos la desarrollan a muy temprana edad y otros en la edad adulta [94, 95]. Por tanto, ser portador de los heterodímeros HLA-DQ2/8 es necesario pero no suficiente para padecer la enfermedad, y otros factores ambientales y genéticos están involucrados en el desarrollo de la EC [95].

4.1. Prevalencia y epidemiología

La primera publicación que probó la verdadera magnitud de la EC se debe a Catassi y *col.* en 1996 [96]. En dicha publicación la prevalencia de EC en niños de edad escolar fue de 1:184 y el ratio de casos de EC diagnosticados y no diagnosticados fue 1:7. Los estudios de rastreo en EC pronostican que la enfermedad afecta a un 1% de la población de Estados Unidos y que se desarrolla tanto en niños como en adultos [97]. De todos estos individuos un 10-15% ha sido diagnosticado o tratado. Finlandia tiene uno de los más altos índices documentados, con una estimación que sugiere una prevalencia de un 2% [98]. No existe una asociación significativa entre la prevalencia de EC y consumo de trigo, la suma de las frecuencias de DR3-DQ2 y DR4-DQ8, o el producto de ambos factores, lo que sugiere que otros factores ambientales y genéticos deben estar contribuyendo al

desarrollo o patogénesis de la EC [99]. A pesar de ello, la EC sigue estando infradiagnosticada, y debido a la heterogeneidad de los síntomas de la EC, los investigadores han propuesto representar la EC como un iceberg que define el espectro clínico y patológico de la enfermedad, donde los pacientes no diagnosticados representan la parte sumergida del iceberg [96, 99, 100].

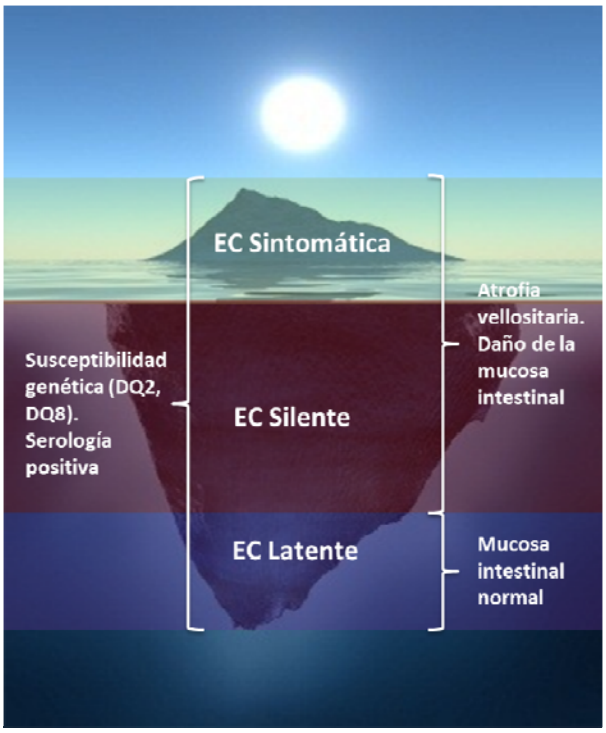

Figura 3. Iceberg de la enfermedad celíaca

4.2. Manifestaciones clínicas y clasificación

Las manifestaciones clínicas de EC varían enormemente, y dependen de la edad de presentación. Los síntomas gastrointestinales y signos de malabsorción son más comunes en niños diagnosticados en los primeros años de vida. A medida que avanza la edad de diagnóstico, aunque también pueden aparecer las típicas manifestaciones digestivas, aparecen síntomas extraintestinales, como anemia ferropénica refractaria, dispepsia, estreñimiento, infertilidad, etc.

4.2.1. Enfermedad celíaca clásica

Es la forma de EC más típica y más fácil de diagnosticar, que consiste en malabsorción, diarrea crónica y distensión abdominal. Se suele presentar en niños en sus primeros años de vida y en algunos adultos. Los pacientes presentan además de la típica sintomatología, atrofia de

vellosidades intestinales y anticuerpos antiendomisio (AEM) y anti-transglutaminasa tisular (ATGt) séricos positivos [101, 102].

4.2.2. Enfermedad celíaca atípica

Los pacientes que presentan esta forma de EC no poseen los típicos síntomas gastrointestinales, sino que desarrollan otro tipo de sintomatología, pero presentan atrofia de vellosidades intestinales y anticuerpos AEM y ATGt séricos positivos. Algunos síntomas extraintestinales son secundarios a la malabsorción, como neuropatía periférica (deficiencia de vitamina B12 y B1), anemia ferropénica (deficiencia de hierro, vitamina B12 y ácido fólico), dolor óseo (osteoporosis y osteopenia, deficiencia de vitamina D y calcio), calambres musculares (deficiencia de calcio y magnesio), ceguera nocturna (deficiencia vitamina A), hemorragia y hematoma (deficiencia vitamina K). Otros síntomas extraintestinales no son secundarios a la malabsorción, como trastornos neurológicos (depresión, epilepsia, migraña, ataxia), dermatitis herpetiforme, hepatitis crónica, infertilidad, estomatitis, nefropatía por IgA, miocarditis. También existen enfermedades asociadas como enfermedades autoinmunes (DT1, síndrome de Sjögren, tiroiditis, hepatitis autoinmune, cirrosis biliar primaria, artritis reumatoide, psoriasis), déficit selectivo de IgA, síndrome de Down, síndrome de Turner, etc. [101, 102].

4.2.3. *Enfermedad celíaca asintomática*

Se caracteriza porque dichos pacientes no presentan ningún tipo de síntoma. Existen dos formas, la **EC silente** y la **EC latente/potencial**. Los pacientes con EC silente no presentan síntomas, pero en cambio, sí presentan atrofia intestinal y anticuerpos AEM y ATGt séricos positivos. Suele ser común en sujetos con historia familiar de EC, o pacientes con enfermedades autoinmunes o genéticas asociadas. Los pacientes con EC latente o potencial tienen una histología normal sin atrofia de vellosidades, pero presentan incremento de LIEs, y la mayoría, anticuerpos AEM y ATGt séricos positivos. Con el tiempo, sin una dieta libre de gluten (DLG), dichos pacientes pueden llegar a desarrollar atrofia intestinal.

4.2.4. Enfermedad celíaca refractaria

La EC refractaria (ECR) se define por persistentes o recurrentes síntomas de malabsorción y atrofia vellositaria intestinal con hiperplasia de criptas, y un incremento de LIEs, a pesar de una estricta DLG durante al menos 6-12 meses, en ausencia de otras causas que lleven a la falta de respuesta al

tratamiento de EC y malignidad manifiesta [103, 104]. Existen dos tipos de ECR, la de tipo 1 que se caracteriza por la presencia de LIEs con fenotipo normal, y la de tipo 2 con presencia de una población clonal de LIEs con un fenotipo anormal con expresión de CD3e intracitoplasmático, CD103 de superficie, y ausencia de los clásicos marcadores de superficie de células T tales como CD8, CD4 y TCR-αβ [104].

4.3. Diagnóstico y tratamiento

Debido a la existencia de diferentes formas clínicas de EC de diferentes sintomatologías, la biopsia intestinal es el estándar de oro en el diagnóstico de la enfermedad. Aun así, los tests serológicos son fundamentales para determinar si el paciente debe someterse a una endoscopia y biopsia. Los anticuerpos anti-gliadina IgA e IgG no son de uso extendido por su falta de sensibilidad y especificidad. En cambio, los anticuerpos AEM siguen siendo el test más específico, aunque el anticuerpo para su auto-antígeno (ATGt) es el más extensamente usado por su alta sensibilidad, especificidad y facilidad para llevarlo a cabo [105].

La DLG es el único tratamiento médico aceptado para la EC. Mejoran los síntomas, la calidad de vida, el intestino llega a tener una biopsia normal y los niveles de anticuerpos se normalizan. Después del diagnóstico, se deben evaluar las posibles deficiencias de vitaminas y minerales y tratarlas con una suplementación adecuada.

5. INMUNOPATOGÉNESIS DE LA ENFERMEDAD CELÍACA

Muchos de los mecanismos moleculares que dan lugar al daño tisular y la respuesta inmunológica en la EC se desconocen. En ellos están implicados la respuesta adaptativa, innata y humoral interaccionando entre sí, dando lugar al estado patológico de la enfermedad.

5.1. Respuesta inmune adaptativa y humoral

Las células T CD4+ específicas de gluten pueden aislarse de pacientes celíacos pero no de individuos sanos [106]. En pacientes celíacos los péptidos derivados del gluten que son presentados en el contexto de las moléculas HLA-DQ2 y HLA-DQ8 inducen una respuesta por parte de las células T CD4+. Estas dos moléculas tienen preferencia por péptidos cargados negativamente, pero los péptidos nativos del gluten no poseen residuos con carga negativa. La enzima transglutaminasa tisular (TGt) se encarga de convertir estos péptidos no cargados en péptidos cargados negativamente, transformando la glutamina (sin carga) a ácido glutámico (con carga negativa), este proceso se denomina deamidación. El gluten es un buen sustrato para la TGt, la cual convierte los residuos de glutamina, que se encuentran en la secuencia glutamina-X-prolina (donde X se refiere a cualquier aminoácido), a ácido glutámico. Cuando los péptidos adquieren carga negativa se unen con mayor afinidad a posiciones fijas de las hendiduras con carga positiva de las moléculas HLA-DQ2 y HLA-DQ8 (P4, P6 y P7 en DQ2, y P1 y P9 en DQ8). Dichas hendiduras se encuentran en las cadenas α y β de las moléculas HLA-DQ2 y HLA-DQ8 [106-109].

La presentación de los péptidos deamidados del gluten a las células T CD4 tiene lugar en el contexto de HLA-DQ2/8 por parte de las células presentadoras de antígeno (APC). La posterior liberación de citoquinas pro-inflamatorias, en particular IFN-γ, provoca una profunda remodelación tisular, y podría activar los linfocitos intraepiteliales a través de la liberación de IL-15 [109].

Un hecho que aun no está claro es, cómo la TGt pasa de su forma inactiva a su forma activa. Tjon *y col.* [110] sugieren que mientras la respuesta de las células T CD4+ contra péptidos nativos del gluten es relativamente rara, puede representar la primera brecha en la tolerancia oral hacia el gluten y la presentación de péptidos nativos del gluten por parte de las moléculas HLA-DQ2/8 a las células T CD4+ podría llevar a la producción de IFN-γ. El IFN-γ produciría el daño tisular que da

lugar a la liberación y la activación de la TGt que modificaría los péptidos nativos a péptidos deamidados. El IFN-γ podría también inducir una mayor expresión de moléculas HLA-DQ.

Después de la presentación de péptidos deamidados del gluten a las células T CD4+ por parte de las APC en el contexto de HLA-DQ2/8, estas células T CD4+ específicas de gluten pueden estimular la producción de anticuerpos anti-gluten y ATGt por parte de las células B [111]. Shiner y Ballard en 1972 [112] fueron los primeros en publicar la existencia de depósitos de IgA en la membrana basal de las células epiteliales de superficie, en el epitelio de las criptas intestinales, alrededor de los fibroblastos subepiteliales, y en las paredes de los vasos sanguíneos de la mucosa intestinal, y luego fue corroborado por otros estudios [113, 114]. Los depósitos de IgA se han encontrado también en la piel y en el cerebro promoviendo respectivamente la dermatitis herpetiforme [115] y la ataxia producida por el gluten [116]. No está claro si los anticuerpos IgA contra el gluten o contra el auto-antígeno TGt son productos secundarios de la respuesta immune adaptativa del intestino o juegan un papel directo en la patogénesis de la EC [117].

5.2. Linfocitos intraepiteliales: entre las respuestas inmunes adaptativa e innata

La mayoría de los LIEs son células T CD8+ TCRαβ+ (75% CD8+ TCRαβ+ y 15% CD8+ TCRγδ) [117]. En EC activa el número de LIEs (células T CD8+ TCRαβ y TCRγδ) se ve incrementado, y expresan altos niveles de receptores NKG2C y NKG2D [118], y las células del epitelio intestinal tienen también una alta expresión de los ligando de receptores NKG2C y NKG2D (MICA y HLA-E) [119]. La interacción de los receptores NK con sus ligandos da lugar a la muerte de las células epiteliales del intestino y la liberación de IFN-γ y proteínas citolíticas (perforinas, grancimas). Todo esto lleva al típico daño tisular. Parece ser que la IL-15 participa en este proceso, ya que se ha visto que estimula la expresión de los receptores NK, NKG2C y NKG2D, en los LIEs de pacientes celíacos e incrementa la capacidad de estos de lisar los enterocitos [111, 119]. Además, en biopsias de EC tratadas con IL-15 existe también una expresión más alta de moléculas MICA [119].

Por tanto, el mecanismo hipotético es que en individuos sanos los LIEs TCRγδ+NKG2A+ inhibirían a los LIEs CD8+TCRαβ+HLA-E+ mediante la secreción de TGF-β. Por el contrario, en EC activa, la liberación de IL-15 por parte del epitelio afectaría de forma negativa a la inhibición producida por los LIEs TCR γδ+ vía TGF-β, aumentaría las señales estimuladoras producidas vía receptor de células T, estimularía la expresión de receptores NK y sus ligandos, y estimularía las funciones efectoras de los LIEs, lo que provocaría el daño del tejido epitelial [117].

5.3. Respuesta inmune innata

Algunos péptidos del gluten, como p31-43/49, pueden inducir daño tisular directo activando el mecanismo de la respuesta inmune innata a través de la inducción de la producción de IL-15 [120]. Meresse *y col.* mostraron que la IL-15 aumenta la expresión de NKG2D en los LIEs, y puede alterar la función del receptor NK dando lugar a una respuesta citotóxica independiente de receptor de células T, mediada por receptores NK por parte de los LIEs [121].

Algunos autores proponen que el hecho de que el péptido p31-43/49 pudiera activar el SI local implicaría que debería existir un receptor para este péptido. Tjon *y col.* evaluaron la hipótesis de la existencia de un receptor para p31-49 en las células del epitelio intestinal. No pudieron detectar la unión de p31-p49 a líneas celulares intestinales, pero los autores hacen hincapié en que en ausencia de un receptor a partir del cual el péptido pueda ejercer su actividad, el mecanismo molecular subyacente a los efectos biológicos observados con estos péptidos permanece sin aclarar [110].

Matysiak-Budnik *y col.* [122] plantearon como hipótesis el transporte del péptido p31-43/49 gracias a los anticuerpos IgA secretora (SIgA) anti-gliadina. Los péptidos del gluten formarían un complejo con la SIgA intraluminal, el complejo se une a un receptor IgA y es transportado por un mecanismo específico de retrotranscitosis, protegidos de la degradación lisosomal. Proponen como receptor implicado en la retrotranscitosis de IgA en la EC al receptor de transferrina CD71, ya que en el intestino de individuos sanos y pacientes con una DLG, CD71 se expresa solamente en la membrana basolateral del enterocito y en pacientes con EC activa la expresión de CD71 está aumentada y CD71 se encuentra en la membrana apical del enterocito donde se co-localiza con IgA [122].

6. INFLUENCIA DE LOS FACTORES AMBIENTALES Y GENÉTICOS EN EL DESARROLLO DE LA ENFERMEDAD CELÍACA.

El gluten es el principal factor ambiental responsable de los indicios y síntomas de la enfermedad, pero además otros factores ambientales parecen estar implicados en el riesgo a padecer EC. Existen también otros genes, diferentes a los genes HLA-DQ, que deben explicar el riesgo genético total de desarrollar EC. Además, también se piensa que la interrelación entre los factores ambientales y el componente genético, juega un papel en el riesgo a padecer la enfermedad, incluyendo el tipo de lactancia, incidencia de infecciones, y disbiosis intestinal, entre otros [123].

6.1. Riesgo genético

Mediante los estudios de asociación del genoma completo (del inglés, GWAS (*genome-wide-association studies*), se han podido detectar algunos de los genes que explican el restante 60% del riesgo genético de EC y los genes que la EC comparte con otras enfermedades autoinmunes. Mediante estas dos aproximaciones los investigadores intentan encontrar los genes involucrados en la enfermedad y los mecanismos biológicos subyacentes a su desarrollo.

Dubois y col. llevaron a cabo un GWAS y encontraron 13 nuevas regiones de riesgo de EC. Describieron 4 rutas inmunológicas específicas que son relevantes en la patogénesis de la EC, y en las que participaban genes incluidos en las 13 regiones de riesgo. Estas 4 rutas están involucradas en: (1) el desarrollo de células T en el timo, (2) detección inmunológica innata de ARN viral, (3) coestimulación de células T y B, y (4) producción de citoquinas, quimioquinas y sus receptores.

La EC ha sido asociada a otras enfermedades autoinmunes, particularmente a la DT1, y la artritis reumatoide (AR) [95, 99]. La EC, la DT1 y la AR comparten la asociación con la región HLA y otras regiones no HLA, incluidas las regiones IL2-IL21, SH2B3, y TAGAP [124, 125]. En los últimos años, ha aumentado la incidencia de EC y DT1 [124], y además se ha sugerido que la ingesta de gluten, y la permeabilidad e inflamación intestinal son factores involucrados en el desarrollo de DT1 [126]. Por otra parte, aunque la EC y la AR sean distintas en su genotipo, poseen características comunes como infiltración de células T en órganos diana, el desarrollo de auto-anticuerpos, y la existencia de enzimas involucradas en la patogénesis de la enfermedad [125].

Aunque poco a poco se van descifrando algunas interacciones entre factores ambientales y genéticos, y que además dichas interacciones son compartidas por varias enfermedades autoinmunes, son necesarios más estudios sobre estas interacciones para establecer los mecanismos biológicos subyacentes al desarrollo de la EC.

6.2. Lactancia materna

El papel protector de la leche materna frente al desarrollo de EC ha sido revisado y analizado en un meta-análisis de estudios retrospectivos observacionales, que concluyó en que el incremento de la duración de la lactancia materna está asociado a la reducción del riesgo de EC [127]. Cinco de los seis estudios que habían satisfecho el criterio de inclusión de calidad metodológica, encontraron que los niños con EC habían sido amamantados por un periodo significativamente menor comparado con los controles. La edad al tiempo de la evaluación del riesgo fue variable entre los participantes y entre los estudios, desde menos de 2 años hasta una media de 7,9 años. El meta-análisis de 4 de estos estudios llevó a la conclusión de que el riesgo a desarrollar EC se redujo significativamente en los niños que se les introdujo el gluten en la alimentación al mismo tiempo que estaban siendo alimentados con leche materna (OR: 0,48; 95%CI: 0,40-0,59). Sin embargo, los estudios revisados no dejan claro si la lactancia materna solamente retrasa la aparición de los síntomas o proporciona protección permanente frente a la enfermedad. Por otra parte, los resultados del meta-análisis están sujetos a limitaciones, como las derivadas del sesgo a la hora de recordar, por ejemplo, la duración de la lactancia materna y la edad de introducción del gluten. Además otra fuente de sesgo podría derivar de un ajuste sub-óptimo de los posibles factores de confusión dentro de los niños alimentados con leche materna y los que no. Por ejemplo, solo uno de los estudios controló por genotipo HLA, que, casualmente, fue el único estudio que no encontró una relación entre lactancia materna y protección frente a EC. Dadas estas limitaciones, parece claro que se requieren estudios de cohorte prospectivos a largo plazo para investigar más a fondo la relación entre lactancia materna y EC.

El estudio de Ivarsson *y col.* [128], incluido en el meta-análisis, es un estudio de caso-control de base poblacional de 627 casos con EC confirmada (registro de EC entre Noviembre de 1992 y Abril de 1995) y 1254 controles, en el que se evaluaron los patrones de introducción de alimentos en niños. El estudio reveló que el riesgo de EC se redujo en niños suecos menores de 2 años si estaban todavía siendo amamantados cuando se les introdujo el gluten en la alimentación (OR: 0,59; 95%CI: 0,42-0,83), y el riesgo aumentó cuando se introdujo el gluten en grandes cantidades (OR: 1,5; 95%CI: 1,1-2,1). Es biológicamente probable que el consumo de la leche materna al mismo tiempo que se introduce el gluten constituya una oportuna ventana temporal para que se desarrolle la tolerancia oral a los antígenos de importancia. En este estudio, la exposición a los factores de riesgo explorados no tuvo o fue de menor importancia en los niños mayores de dos años. Por tanto, es importante también investigar si los patrones dietéticos favorables posponen el desarrollo de EC o reducen el riesgo total a lo largo de la vida [128].

Previo a este análisis de factores de riesgo, los autores habían informado de una epidemia de EC sintomática entre 1984 y 1996 en niños suecos menores de 2 años de edad, parcialmente explicada por cambios en la alimentación de los niños [129]. El incremento de la incidencia fue precedido de un incremento en la cantidad de gluten consumido y por el retraso en la introducción del gluten en la dieta, lo cual podría haber dado lugar a una mayor proporción de niños a los que se les daba grandes cantidades de gluten cuando ya se le había retirado la lactancia materna. Además un estudio realizado en el año 2009 sobre la prevalencia de EC en niños suecos nacidos en 1993, durante la epidemia, y bajo las nombradas prácticas dietéticas desfavorables, desveló que la prevalencia fue de un 3%, 3 puntos más alta que la prevalencia indicada normalmente del 1% [130].

Existen estudios que muestran que el retraso de la introducción de la leche de vaca que acontece cuando la lactancia materna es más prolongada reduce el riesgo de desarrollar DT1 en la infancia [131]. Parece plausible que además de los componentes inmunológicamente activos en la leche materna, el evitar la introducción temprana de las proteínas de la leche de vaca, contribuye también al efecto protector de la leche materna. Este podría ser también el caso de la prevención de EC mediante la lactancia materna. Además, la diferencia en la microbiota intestinal de niños alimentados con leche materna y leche de fórmula, también podría explicar la protección frente al desarrollo de EC observada con la lactancia materna [132].

6.3. Infecciones

Debido a que el IFN-γ y la IL-15 juegan papeles clave en las respuestas tempranas a patógenos intracelulares, se ha contemplado la capacidad de los agentes infecciosos para actuar como factor desencadenante del mecanismo inmunopatogénico que da lugar a la ruptura de la tolerancia oral. De hecho, los virus de ARN de doble cadena son potentes inductores de ambas citoquinas, y podrían además re-instruir o reclutar *de novo* y activar CDs para imprimar a las células T específicas de gluten [117]. En EC, se ha sugerido que la exposición a agentes infecciosos (virus y bacterias) puede ser un factor causante del daño tisular e inflamación, que puede contribuir con el tiempo a reducir la tolerancia al gluten [133].

En algunos estudios, aunque no todos, el análisis de anticuerpos en suero mostró asociación entre desarrollo de EC y infecciones pasadas por adenovirus tipo 12 [134, 135] y hepatitis C [135]. Se ha sugerido también que el mimetismo molecular de las proteínas virales con péptidos tóxicos del gluten podrían modular la tolerancia inmunológica del huésped y desencadenar el desarrollo de EC [135, 136]. Sin embargo, existen otros estudios que apoyan el papel protector de las infecciones frente al desarrollo de EC. Plot *y col.* [137] analizaron los anticuerpos específicos

de patógenos en suero de pacientes celíacos y lo compararon con el de gente sana. Examinaron la asociación de EC con 5 agentes infecciosos, *Toxoplama gondii*, virus de la rubeola, citomegalovirus, *Treponema pallidum*, y virus de Epstein-Barr. Los resultados serológicos demostraron una menor prevalencia de anticuerpos IgG en pacientes celíacos comparado con los sujetos sanos. Los estudios basados en la detección de anticuerpos frente a virus en el suero de los pacientes en comparación con los controles son contradictorios en relación con la asociación entre la infección y el desarrollo de EC [138, 139].

Los patógenos gastrointestinales como el rotavirus se han asociado también a EC. Evidencias epidemiológicas y prospectivas apoyan el papel de las infecciones múltiples por rotavirus en el incremento del riesgo a desarrollar EC en individuos genéticamente predispuestos [140], posiblemente a través de la lesión de la barrera intestinal y la facilitación de la penetración de los epítopos tóxicos de gliadina.

6.4. Microbiota intestinal

Recientemente se ha relacionado también la composición de la microbiota intestinal con la EC. Las primeras asociaciones se establecieron en pacientes celíacos tratados y no tratados con DLG. La disbiosis intestinal de pacientes celíacos se caracterizó por un incremento en número y proporción de *Bacteroides* spp., y una reducción de *Bifidobacterium* spp. y *Bifidobacterium longum*, que no se normalizó completamente después de la adherencia del paciente a la DLG [141-143]. Los números de *Escherichia coli* y *Staphylococcus* fueron también más altos en heces y biopsias de pacientes celíacos no tratados comparado con los controles, pero las diferencias se normalizaron después de la retirada del gluten [142]. El análisis de la prevalencia de especies bacterianas asociadas a biopsias duodenales reveló una reducción en la diversidad de especies de *Bacteroides* en pacientes tratados y no tratados con DLG, en comparación con los controles [144]. Estos resultados indican que la disbiosis intestinal está asociada a la EC.

Continuando con estos estudios, se están llevando a cabo otros para establecer los posibles efectos del tipo de lactancia y el genotipo HLA-DQ sobre la microbiota a edades tempranas, y la posible influencia sobre el desarrollo de EC [145, 146]. En este contexto, un estudio prospectivo en el que se incluyó a 164 recién nacidos sanos, con un familiar de primer grado celíaco, indicó que tanto el tipo de lactancia como el genotipo HLA-DQ tenían efecto sobre el establecimiento de la microbiota intestinal de los niños [146]. Se encontró un número reducido de *Bifidobacterium* spp. en niños con mayor riesgo de desarrollar EC, tanto en niños alimentados con leche materna como en los alimentados con leche de fórmula. En la población global la lactancia materna

favoreció la colonización de ciertas especies de este género, como *B. longum* y *B.breve*. El mayor riesgo a desarrollar EC se asoció con el incremento en el número de *Staphylococcus* spp., tanto en la población total como en los subgrupos de leche materna y leche de fórmula, pero la colonización por este grupo bacteriano no se vio favorecida por la lactancia de fórmula sin tener en cuenta el riesgo, sugiriendo que el genotipo HLA-DQ juega un papel más importante en su colonización. Se encontró también un mayor número de *Bacteroides fragilis* en niños con alto riesgo genético. Sin embargo, está asociación solo se confirmo en el grupo de niños alimentados con leche de fórmula y no en el de lactancia materna, indicando que el tipo de lactancia es el principal factor que influye en la colonización por este grupo bacteriano [146]. Otro estudio sobre la prevalencia de Bacteroides spp. en un subgrupo dentro de la cohorte de niños del estudio anterior, mostró que la prevalencia de *Bacteroides vulgatus* fue mayor en niños con alto riesgo genético de EC, mientras que la prevalencia de *Bacteroides uniformis* fue mayor en niños alimentados con leche materna y con un bajo riesgo genético [145]. En general, la lactancia materna parece reducir las diferencias en la microbiota relacionadas con el genotipo HLA-DQ, lo que puede explicar en parte el efecto protector de la leche materna observado en algunos estudios observacionales previos [127, 128].

OBJETIVOS

Los componentes ambientales y genéticos que intervienen en la EC son muy complejos y gran parte de ellos se desconocen. El actual trabajo forma parte del estudio PROFICEL, el cual va dirigido a descubrir qué factores ambientales pueden estar implicados en el desarrollo de EC en individuos con susceptibilidad genética, y su posible prevención a través de potenciales probióticos.

Teniendo en cuenta esto, el primer objetivo de este trabajo ha sido estudiar el efecto conjunto del genotipo HLA-DQ y de diversos factores ambientales sobre el desarrollo del SI y el establecimiento de la microbiota intestinal en niños con riesgo familiar de EC, para en un futuro poder identificar, con el seguimiento prolongado de este grupo de niños, los principales factores que contribuyen a la susceptibilidad de padecer EC.

Un segundo objetivo ha sido evaluar *in vitro* la capacidad de inmunomodulación de diferentes cepas bacterianas intestinales características de niños alimentados con leche materna y leche de fórmula, para contribuir a identificar posibles cepas bacterianas probióticas que puedan resultar beneficiosas en el contexto de la EC.

Para ello, se han seguido los siguientes objetivos parciales:

1. Estudiar la influencia de la lactancia materna vs. fórmula sobre las poblaciones linfocitarias de niños con riesgo de desarrollar EC (cohorte del estudio PROFICEL).

2. Estudiar la influencia de diversos factores ambientales sobre las poblaciones linfocitarias y la microbiota intestinal de niños con riesgo de desarrollar EC (submuestra de la cohorte PROFICEL).

3. Estudiar el efecto inmunoestimulador *in vitro* de diferentes cepas bacterianas intestinales características de niños alimentados con leche materna y leche de fórmula sobre CMNs, y en sistemas de co-cultivo CMNs/células Caco-2

RESULTADOS: ESTUDIOS REALIZADOS

1. Papel de los factores ambientales tempranos en la inmunocompetencia y el proceso de colonización intestinal en lactantes con riesgo familiar de EC

I. Influence of breastfeeding versus formula feeding on lymphocyte subsets in infants at risk of coeliac disease: the PROFICEL study [147].

II. Influence of early environmental factors on lymphocyte subsets and gut microbiota in infants at risk of coeliac disease: the PROFICEL study (aceptada en *Nutrición Hospitalaria*).

I. Influence of breastfeeding versus formula feeding on lymphocyte subsets in infants at risk of coeliac disease: the PROFICEL study [147].

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(ver ANEXO I)

RESUMEN

Propósito Además del riesgo genético, los factores ambientales podrían influir en el desarrollo de la EC. Hemos tratado de evaluar el efecto de la interacción entre el tipo de lactancia y el genotipo HLA-DQ sobre las subpoblaciones de linfocitos aislados de sangre periférica, y sus marcadores de activación, en niños con riesgo familiar de EC.

Métodos 170 niños fueron clasificados en 3 grupos diferentes de acuerdo a su riesgo genético (riesgo alto, RA; riesgo intermedio, RI; y riesgo bajo, RB) después de haber realizado el tipaje HLA DQB1 Y DQA1. Las subpoblaciopnes de linfocitos se estudiaron a los 4 meses de edad mediante citometría de flujo.

Resultados 79 niños recibieron lactancia materna exclusiva (LM), y 91 niños lactancia mixta o lactancia de fórmula (LF). De acuerdo al riesgo genético, 41 niños se clasificaron en el grupo RA, 75 en el grupo RI, y 55 en el grupo RB. La ANOVA de 2 vías no mostró interacciones significativas entre el tipo de lactancia y el grupo de riesgo genético sobre las subpoblaciones de linfocitos analizadas. La ANOVA de una vía para el tipo de lactancia mostró que el porcentaje de células CD4+CD25+ fue significativamente mayor en el grupo LM que en el grupo LF (LM, 10,92 ± 2,71; LF, 9,94 ± 2,96; p=0,026), y el número absoluto de células CD4+CD38+ fue significativamente mayor en el grupo LF que en el grupo LM (LF, 2881,23 ± 973,48; LM, 2557,95 ± 977,06; p=0,038). La ANOVA de una vía para el riesgo genético mostró que el número absoluto de células NK fue significativamente mayor en el grupo RI que en los grupos RA y RB (RI, 539,24 ± 340,63; RA, 405,01 ± 239,53; RB, 419,86 ± 262,85; p=0,028).

Conclusión En las primeras etapas de vida los perfiles de subpoblaciones de linfocitos podrían ser modulados por el tipo de lactancia y el riesgo genético separadamente. La lactancia materna podría tener un efecto inmunomodulador positivo sobre las subpoblaciones de linfocitos de niños con riesgo a desarrollar EC.

ABSTRACT

Purpose In addition to genetic risk, environmental factors might influence coeliac disease (CD) development. We sought to assess the effect of the interaction between milk-feeding practices and the HLA-DQ genotype on peripheral lymphocyte subsets and their activation markers in infants at familial risk for CD.

Methods 170 newborns were classified in 3 different genetic risk groups (high risk, HR; intermediate risk, IR; and low risk, LR) after DQB1 and DQA1 typing. Lymphocyte subsets were studied at the age of 4 months by flow cytometry analysis.

Results 79 infants were receiving exclusive breastfeeding (BF) and 91 partial breastfeeding or formula feeding (FF). Regarding genetic risk, 40 infants were classified in HR group, 75 in IR group and 55 in LR group. Two-way ANOVA did not show significant interactions between the type of milk feeding and genetic risk group on the lymphocyte subsets analysed. One-way ANOVA for milkfeeding practice alone showed that the percentage of CD4+CD25+ cells was significantly higher in BF group than in FF group (BF, 10.92 ± 2.71; FF, 9.94 ± 2.96; p=0.026), and absolute counts of CD4+CD38+ cells were significantly higher in FF group than in BF group (FF, 2,881.23 ± 973.48; BF, 2,557.95 ± 977.06; p=0.038). One-way ANOVA for genetic risk alone showed that absolute counts of NK cells were significantly higher in IR group than HR and LR groups (IR, 539.24 ± 340.63; HR, 405.01 ± 239.53; LR, 419.86 ± 262.85; p = 0.028).

Conclusion Lymphocyte subset profiles in the early stages of life could be modulated by milk-feeding practices and genetic risk separately. Breastfeeding might have a positive immunomodulatory effect on lymphocyte subsets in infants at risk of CD.

Keywords: Lymphocyte subsets; Coeliac disease; Infants; HLA genotype; Breastfeeding; Formula feeding.

Abbreviations:

CD	Coeliac disease
PCR-SPP	Polymerase chain reaction-sequence-specific primers
HLA	Human leucocyte antigen
Treg	Regulatory T cells
NK	Natural killer
LR	Low genetic risk
IR	Intermediate genetic risk
HR	High genetic risk
BF	Breastfeeding
FF	Formula/mixture feeding

INTRODUCTION

Coeliac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of gluten in genetically susceptible individuals. It is generally accepted that CD is a T-cell-mediated disease, in which gliadin-derived peptides activate lamina propria infiltrating T lymphocytes. The subsequent release of pro-inflammatory cytokines, in particular γ-interferon, leads to a profound tissue remodelling. However, an innate immune response also seems to be involved in the disease development [1]. This is a complex disorder, with environmental and genetic factors contributing to its aetiology. The major genetic risk factor in CD is represented by HLA-DQ genes. Several studies have documented that the HLA-DQA1*05 and DQB1*02 alleles, encoding for particular DQ2 molecules, confer high susceptibility to CD. This heterodimer can be encoded both in cis and in trans. The susceptibility to CD is increased in homozygous subjects with a cis haplotype or possessing a second HLA-DQB1*02 allele [2–4]. In Europe, approximately 90 % of patients have these genetic markers, whereas most of the remaining cases carry the HLADQA1* 03 and DQB1*0302 alleles coding for DQ8 molecules [3, 5]. Gluten is the main environmental factor responsible for the signs and symptoms of the disease. Moreover, the interplay between other environmental elements and the genetic background is also thought to play a role in the disease risk, including the type of milk feeding, incidence of infections and intestinal dysbiosis [6].

The effect of environmental factors on future disease risk is relevant at the early stages of life when the immature neonate's gut undergoes the process of microbiota establishment and the immune system acquires full competence and tolerance to nonharmful antigens [7]. A particular feature of the neonatal immune system is the presence of a wide pool of naı¨ve T cells waiting to participate in primary immune responses. Intestinal antigen exposure during neonatal life influences appropriate adult immune responses. T-cell differentiation occurs within the neonatal human intestine, and the T-cell receptor (TcR) repertoire of these developing immature T cells is likely to be influenced by luminal antigens [8]. Oral ingestion of food and environmental bacteria is a major route of antigenic exposure. After birth, lactation supports immunological defences through a number of molecules with antimicrobial activity and immunologically active components present in breast milk [9, 10]. Thus, breastfeeding is known to confer protection against infectious diseases in the short term and there is also some evidence of lower prevalence of inflammatory bowel diseases, childhood cancers and type I diabetes in breastfed infants [11].

As early events during extra-uterine life allow the maturation of the immune system, the peripheral lymphocyte subsets also exhibit certain changes. Blood cell analysis shows marked lymphocytosis at birth and at later stages of life compared to adulthood. An increase in T and B lymphocytes occurs during the first weeks of life, while NK cells decline sharply directly after birth

[12, 13]. A high CD4/CD8 ratio in babies up to 2 years of age has been found compared to the values observed in normal adults, and this is mainly due to the large and expanding pool of naïve T CD4+ cells present during the first year of life [14, 15]. The ectoenzyme CD38 is expressed in almost all T and B cells [12] and decreases towards the adult life [15, 16]. Although breastfeeding is likely to contribute to these changes, only a limited number of studies have addressed the influence of milk-feeding practices on peripheral lymphocyte subsets or the immunocompetence of lactating children [17–20] and these findings are not consistent among them.

Taking in consideration how important the maturation of the immune system can be as a predisposing or protective factor in future disease development, we planned to assess the effect of milk-feeding practices on the levels of lymphocyte subsets and their activation markers in infants at risk of CD. Since genetic background is a key issue for disease development in this population, we also aimed to find out possible interactions between type of milk feeding and the HLA genotype on the lymphocyte subsets of these infants.

MATERIAL AND METHODS

Subjects

This study was conducted in 4-month-old infants who are first-degree relatives of CD patients (at least one parent or sibling affected with CD). A total of 170 infants born between October 2006 and the end of 2010 were recruited from 6 hospitals geographically distributed throughout Spain. The 170 infants were divided according to milkfeeding practices into two groups: breastfed (BF) infants (N = 79), including those which had been exclusively breastfed until the age of 4 months, and formula-fed (FF) infants (N = 91), including babies receiving milk formula, either alone or in alternation with breast milk at the moment of analysis. Questions about delivery, infections, vaccinations, feeding practices, clinical symptoms and medical treatments were answered by the parents at the infant's age of 1 and 4 months in clinical visits with the paediatric gastroenterologist. To facilitate the collection of this information, a diary was given to the mothers upon recruitment (before delivery). The study was approved by the local ethic committees, and written informed consent was obtained from the parents of children included in the study.

This cohort of infants was analysed for the HLA class II DQA1 and DQB1 genotypes. The infants were classified into three main risk groups, according to their DQ haplotype, loosely based on the criterion of Bourgey et al. and considering the HLA distribution of the Eastern Spanish population [21]. We established the following risk groups: The first one included those individuals carrying

the DQ2 haplotype, both in *cis* (DQA1*0501-DQB1*0201 in homozygosis) and in *trans* (DQA1*0201-DQB1*0202 with DQA1*0505-DQB1*0301 in heterozygosis) conformation. The second group included those subjects carrying the DQ2 along with any other haplotype, as well as subjects carrying the DQ8 haplotype (DQA1*0301-DQB1*0302) in homozygosis. The third group included those individuals with other common genotypes not associated with CD. Probabilities are approximated and have been estimated taking into account that in most cases, the genotype of the coeliac relative of each newborn was not considered. 40 infants were in the first risk group (high risk; HR) with the highest probabilities of developing CD (28–24 %) [4]. In the second group, with a probability between 7 and 8 %, 75 of the infants were grouped (intermediate risk; IR), and the remaining 55 were in the third group (low risk; LR), with less than 1 % risk of developing CD.

DNA isolation

DNA was extracted from buccal mucosa cells by scraping the inner side of the children's cheek with sterile swabs (Copan innovation, Sarstedt, Germany). The cotton was cut and incubated in DLB buffer (100 mM Tris–HCl pH 7.4, 10 mM EDTA pH 8, 10 mM NaCl per litre of distilled water), 10 % SDS and 10 µL proteinase K (20 mg/ml) at 65 ºC for 1 h, and then, a standard phenol–chloroform method was carried out. Extracted DNA was stored in TE buffer (10 ml Tris–HCl pH 8, 200 µL EDTA 0.5 M pH 8 per litre of distilled water) at -20 ºC after genotyping. The DNA concentration, around 50–100 µg/mL, was quantified using the NanoDrop© Spectrophotometer.

HLA-DQ genotyping

Low-resolution HLA-DQB1 typing was performed by PCR-SSP (polymerase chain reaction-sequence-specific primers) analysis. Each PCR was performed on about 60–90 ng of extracted DNA, 0.5 U of BIOTOOLS DNA polymerase (Biotools B&M S.A, Spain), 1 x PCR Master Mix (Dynal AllSet + TM SSP or Olerup SSPTM) containing nucleotides (200 µmol $^{L-1}$ each), PCR buffer (50 mmol L^{-1} KCl, 1.5 mmol $^{L-1}$ $MgCl_2$, 10 mmol $^{L-1}$ Tris–HCl pH 8.3, 0.001 % w/v gelatine), 5 % glycerol and 100 µg mL^{-1} cresol red, 0.25 µmol $^{L-1}$ of each allele- or group-specific primer pair and 0.1 µmol $^{L-1}$ of internal positive control primer pair matching a segment of the human growth hormone gene in a final volume of 10 µL. Detailed PCR protocol: An initial denaturation step at 94 ºC for 2 min was followed by 10 two-temperature cycles (94 ºC for 10 s and 65 ºC for 60 s) and 20 three-temperature cycles (94 ºC for 10 s, 61 ºC for 50 s and 72 ºC for 30 s). Detection of amplified alleles was carried out by 2 % agarose gel electrophoresis and ethidium bromide staining. Although the

allele DQB1*02 is a determinant of DQ2, HLA-DQA1 alleles were genotyped in a stepwise fashion for a high-resolution typing to sharpen the risk classification of each individual.

Blood analyses

At the age of 4 months, peripheral blood samples were drawn from the infants and collected in vacutainer tubes containing K_3EDTA. Immediately after, 1 mL of blood was mixed with an equal volume (1 mL) of preservative solution (Streck Cell Preservative™ CE. Streck, USA) and sent in cool temperature within 2–6 days to our laboratory for a centralised processing and flow cytometry analysis. Blood extractions were always performed previous to the standardised 4-month vaccination in the Spanish vaccination schedule. Complete blood counts and differential counts were performed in situ at the corresponding enrolling centres by automated instrumentation. Blood samples were not taken if the infant presented an ongoing infection or had received a vaccination shot in the previous 6 weeks.

Flow cytometry analysis

Aliquots of blood mixed with the preservative solution (150 µL) were incubated for 30 min at room temperature and in the dark with 20 lL of fluorochrome-conjugated monoclonal antibodies specific for CD3+ (CD3-APC), CD4+ (CD4-PerCP-Cy5.5), CD8+ (CD8-PerCP-Cy5.5), CD45RA+ (CD45RA-FITC), CD45RO+ (CD45RO-PE), CD25+ (CD25-FITC), HLA-DR+ (HLA-DR-FITC), CD38+ (CD38-PE), in quadruple immunostainings, and 100 µL aliquots were incubated with the multitests CD3-FITC/CD16+56-PE/CD45-PerCP-Cy5.5/CD19-APC and CD3-FITC/CD8-PE/CD45-PerCP-Cy5.5/CD4-APC. Fluorochrome-conjugated isotype control immunoglobulins (IgG1 and IgG2a) from mouse were used for each monoclonal antibody to avoid any background fluorescence signal due to nonspecific binding. All the monoclonal antibodies were purchased from Becton–Dickinson (Sunnyvale, CA, USA). After incubation, samples were lysed with the BD FACSTM Lysing Solution (Becton–Dickinson) following the manufacturer's protocol. The samples were analysed with FACSCalibur Flow Cytometer (four-colour, dual-laser, Becton–Dickinson). The lympho gate was defined on the forward and side scatter patterns of lymphocytes. The analysis protocol gated on lymphocytes stained with PerCP and/or APC, and the selected population was then analysed with the two remaining colours (FITC and PE) to obtain cell percentages expressing the specific antigens. Cell subset counts were obtained by multiplying subset percentages times anchor marker counts, the later resulting by multiplying subset percentage times the absolute lymphocyte count.

Statistics

The normality of the distribution of variables was confirmed through normality graphs and Shapiro–Wilk tests. All variables fitted normal distribution. A univariant analysis of variance was employed with HLA risk and milk-feeding groups as fixed factors to assess the interaction between HLA status and type of milk feeding. When a significant interaction was found, differences between milk-feeding groups were analysed by t test comparison of means within each risk level, and differences between risk groups by one-way ANOVA within each feeding type level followed by post hoc analysis. When no interaction was obtained between the two factors, the analyses were carried out for each factor separately. A p value lower than 0.050 was considered statistically significant.

RESULTS

The demographic characteristics of the infants included in the study are presented in Table 1. All infants were full-term (39.02 ± 1.77 weeks of gestation), and the majority of them were vaginally delivered (118 out of 170). The size and the weight of the infants at the moment of the delivery were within standard ranges and did not differ significantly between the groups.

The percentage and cell counts of the main lymphocyte subsets and subsets expressing activation markers in the whole group of 4-month-old infants are shown in Table 2. Two-way ANOVA for type of milk feeding and genetic risk group did not show significant interactions between genetic risk and milk-feeding practices on the lymphocyte subsets analysed.

Two effects of the milk-feeding type alone were found (Table 3). Firstly, one-way ANOVA for milk-feeding practice alone showed that the percentage of CD4+CD25+ cells was significantly higher in BF group than in FF group (BF, 10.92 ± 2.71; FF, 9.94 ± 2.96; p = 0.026), and secondly, absolute counts of CD4+CD38+ cells were significantly higher in FF group than in BF group (FF, 2,881.23 ± 973.48; BF, 2,557.95 ± 977.06; p = 0.038). The effects of HLA genotype alone were also analysed (Table 4). One-way ANOVA for genetic risk alone showed that absolute counts of NK cells were significantly higher in IR group than HR and LR groups (IR, 539.24 ± 340.63; HR, 405.01 ± 239.53; LR, 419.86 ± 262.85; p = 0.028).

DISCUSSION

In this study, the combined effect of HLA-DQ genotype and milk-feeding practices at 4 month of age on peripheral lymphocyte subsets was studied in a group of infants with at least one first-

degree relative suffering from CD. The infants' HLA genotype was classified into genetic risk groups in concordance with the last results obtained from the Italian and Spanish population distribution [4, 21]. However, taking into account our sample size, the five-group initial classification was reduced to a three-group one. Our analysis did not reveal any interaction between type of feeding and genotype on lymphocyte subsets' profile, but both factors showed an independent effect on specific T-cell subsets. The results obtained for the 20 different lymphocyte subsets measured (median and 10th and 90th percentiles) are in agreement with data published in the literature for 3- to 6-month-old healthy infants [15, 22]. However, no reference data have been published so far for this age range in the Spanish population.

A role for breastfeeding as an environmental factor with the capacity to produce immune modulation that might perhaps delay or reduce the risk of developing CD has been suggested by several authors [23–25]. In our population of infants at risk, we have only found a slight modulation of the lymphocyte subset counts by breastfeeding, since most of the subsets did not show differences between breastfed and formula-fed infants. In the literature, the effects of breastfeeding versus formula feeding on lymphocyte subsets are controversial, but two different studies have found an increased percentage of CD8+ cells in breastfed infants at 6 and 8 months, compared to formula-fed infants [19, 20]. In our infants, no differences were found in the CD4+ and CD8+ lymphocytes; however, we found lower CD4+CD38+ counts and higher CD4+CD25+ percentage in breastfed infants compared to FF infants. CD38+ has a role in T-cell activation and differentiation [26] and is constitutively expressed in newborns decreasing towards the adult life. The fact that lower CD4+CD38+ counts are found in breastfed children supports the enhancing effect of breastfeeding on lymphocyte maturation.

Regarding the lower CD4+CD25+ percentage with formula feeding, this might reflect a lower number of regulatory T cells in these infants. However, since we did not use a specific Treg marker, a major drawback of our study is the impossibility to differentiate within the CD4+CD25+ population what proportion is activated to produce Th1 cytokines and what proportion is Treg to produce IL-10 and TGF-β. The importance of the Treg cells in the susceptibility of atopic disease in children with a familial history of the disease has been pointed out recently [27], even though their role is still not well defined. The modulation exerted by breastfeeding on this population seems relevant, since different animal and human studies have revealed that breast milk induces oral tolerance and prevents asthma and other autoimmune disorders development through an increase in CD4+CD25+ Foxp3+ regulatory T cells [28–30]. Several in vitro studies showed that specific milk components, like exosomes [31], lactobacilli [32] and lactadherine milk protein [33], activate or increase the number of regulatory T cells (CD4+CD25+Foxp3+). Lactadherine, in

addition, has been involved in protection against rotavirus infection [34–37]; some investigations have linked this pathogen to coeliac disease development [38].

Overall, our results showing a percentage of T CD4+CD25+ cells significantly higher in BF group than in FF group, and absolute counts of T CD4+CD38+ cells significantly higher in FF group than in BF group, suggest that breastfed infants could have a more mature immune system that formula-fed infants due to the beneficial properties of human milk. This would be an argument in favour of the recommendation to introduce gluten in predisposed infants while they are still breastfed as a means to possibly lessen the toxicity of gliadin peptides [39]. However, since at the time of our lymphocyte subset analysis, gluten introduction had not occurred yet, it will be interesting to find out whether this apparently increased number of regulatory T-cell clones is associated with a better response to gliadin after gluten introduction. In addition, there are studies that demonstrate that breast milk contains gliadin peptides [40] and gliadin-specific IgA antibodies [40–42] that could be involved in the modulation of the immune response in neonates [40], possibly with a preventive role [42].

Regarding higher absolute counts of NK cells in IR group than HR and LR groups, the possibility exists that this is only a spurious finding, with no biological relevance. However, the association of specific genotypes with a certain marker, a function or a disease, is usually difficult to explain. In this sense, the concept that genetic factors influence the regulation of lymphocyte subpopulations has been supported by several published studies [43–46]. Therefore, more research is needed to clarify the true meaning of the association between NK cells and HLA-DQ genotype in the population studied.

In conclusion, according to our results, the effect of breast milk on lymphocyte subsets could be beneficial in infants at risk of CD and further studies are necessary to assess the combined effect of milk-feeding practices and gluten introduction practices on that risk.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Las tablas y figuras del presente artículo se presentan junto con el artículo original (en formato pdf) que se incluye en el ANEXO I.

II. Influence of early environmental factors on lymphocyte subsets and gut microbiota in infants at risk of celiac disease: the PROFICEL study.

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Pozo-Rubio T *et al.* Nutr Hosp 2012 (aceptada, ver aceptación en ANEXO I)

RESUMEN

Introducción Es bien sabido que el genotipo HLA puede explicar un 40% del riesgo genético de enfermedad celíaca, por lo que otros factores de predisposición genéticos así como factores que sutilmente modulen la activación y diferenciación de células T necesitan ser estudiados. Esto incluye factores ambientales, de los que se cree actualmente que ejercen un efecto sobre el desarrollo del sistema inmune y la microbiota intestinal.

Objetivo Evaluar las asociaciones entre factores ambientales tempranos, las subpoblaciones de linfocitos y la composición de la microbiota intestinal en niños con riesgo familiar de enfermedad celíaca.

Diseño del estudio Estudio prospectivo observacional

Sujetos 55 niños de 4 meses de edad con al menos un familiar celíaco de primer grado. Los niños fueron clasificados de acuerdo al tipo de parto, ingesta materna de antibióticos durante el embarazo y durante el parto, tipo de lactancia, infecciones tempranas y toma de antibióticos, administración de la vacuna de rotavirus, y incidencia de alergias dentro de los 18 primeros meses de vida.

Métodos Las subpoblaciones de linfocitos y la composición de la microbiota intestinal fueron estudiadas a la edad de 4 meses.

Resultados La lactancia de fórmula y las infecciones tempranas se asociaron con un mayor número absoluto de células CD3+, CD4+, CD4+CD38+, CD4+CD28+ y CD3+CD4+CD45RO+ ($P \leq 0.01$). El parto por cesárea y la administración de la vacuna de rotavirus se asociaron a un menor porcentaje de células CD4+CD25+. La toma temprana de antibióticos se asoció y correlacionó con menor número de *Bifidobacterium longum* y mayor número de *Bacteroides fragilis.*

Conclusiones Las infecciones y la toma de antibióticos en los primeros 4 meses de edad son los factores ambientales tempranos más fuertemente y/o frecuentemente asociados a las subpoblaciones de linfocitos y la composición de la microbiota, respectivamente, en niños con riesgo de enfermedad celíaca.

ABSTRACT

Introduction It is known that the HLA genotype can explain about a 40% of the genetic risk of celiac disease (CD), thus, other genetic predisposing factors as well as factors that subtly modulate T cell activation and differentiation need to be studied. This includes environmental factors that are currently believed to impact on the immune system and gut microbiota development.

Aim To assess the associations between early environmental factors (EEF), lymphocyte subsets, and intestinal microbiota composition in infants at familial risk for CD.

Study design Prospective observational study.

Subjects Fifty-five 4 month-old infants with at least a first-degree relative suffering CD. Infants were classified according to type of delivery, mother's antibiotic intake during pregnancy and during labor, milk-feeding practices, early infections and antibiotic intake, rotavirus vaccine administration, and allergy incidence within the first 18 months of life.

Methods Lymphocyte subsets and gut microbiota composition were studied at the age of 4 months.

Results Formula feeding and infant's infections were associated with higher CD3+, CD4+, CD4+CD38+, CD4+CD28+ and CD3+CD4+CD45RO+ counts ($P \leq 0.01$). Infant´s infections were also associated with higher CD4+CD25+, CD4+HLA-DR+ and NK cell counts ($P \leq 0.01$). Cesarean delivery and rotavirus vaccine administration were associated with lower percentage of CD4+CD25+ cells. Infant's antibiotic intake was associated and correlated with lower counts of *Bifidobacterium longum* and higher counts of *Bacteroides fragilis* group.

Conclusions Infant´s infections and antibiotic intake in the first 4 months of life are the EEF more strongly and/or frequently associated to lymphocyte subpopulations and microbiota composition, respectively, in infants at risk of CD.

Keywords: Lymphocyte subpopulations; gut microbiota; infants; early environmental factors; celiac disease's risk.

Abbreviations

CD: celiac disease

EEF: early environmental factors

BF: breast-fed (infants)

FF: formula-fed (infants)

PBMCs: peripheral blood mononuclear cells

OVA: ovoalbumin

INTRODUCTION

Celiac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of gluten in genetically susceptible individuals. It is generally accepted that CD is a T-cell mediated disease, in which gliadin derived peptides activate lamina propria infiltrating T lymphocytes. The subsequent release of pro-inflammatory cytokines, in particular γ-interferon, leads to a profound tissue remodeling. However, an innate immune response with the participation of IL-15 also seems to be involved in the disease development [1, 2]. This is a complex disorder, with environmental and genetic factors contributing to its etiology. The major genetic risk factor in CD is represented by HLA-DQ genes which account for approximately 40% of the genetic risk for CD. Approximately 90-95 % of CD patients express HLA-DQ2 heterodimers, and the remaining 5-10% of patients express the HLA-DQ8 heterodimer [2]. However, only a subset of individuals that express HLA-DQ2 and HLA-DQ8 heterodimers develop CD and, among them, some individuals develop CD very early in infancy after their first exposure to gluten, and others in adulthood; the reasons for these variations are still unclear [2,3]. Thus, HLA-DQ2/8 heterodimers are necessary but not sufficient, and other genetic and environmental factors must be involved in CD development [3]. Gluten is the main environmental factor responsible for the signs and symptoms of the disease, but also, other environmental elements might play a role in the disease risk [4].

The effect of environmental factors on disease risk is relevant at the early stages of life when the immature neonate's gut undergoes the process of microbiota exposure and establishment, and the immune system acquires full competence and tolerance to non-harmful antigens [5]. A particular feature of the neonatal immune system is the presence of a wide pool of naïve T cells waiting to participate in primary immune responses. In addition, intestinal antigen exposure during neonatal life, and the establishment of the microbiota, influences appropriate adult immune responses [6]. If appropriate immune tolerance is not established in early life and maintained throughout life, this represents a risk factor for the development of inflammatory, autoimmune, and allergic diseases [7]. CD development is also likely to be influenced by factors that modulate the development and maturation of the immune system in infants.

There are several factors influencing the colonization pattern and establishment of microbiota in infants. The microbes colonizing the infant's gut come primarily from the mother's vaginal and perineal microbiota if vaginally delivered, and from the feeding route, breast feeding or bottle feeding. Thus, the colonization pattern is influenced by delivery and feeding modes, and the exposure to pathogens and antimicrobials [8].

As early events during extra-uterine life allow the maturation of the immune system, the peripheral lymphocyte subsets exhibit certain changes. Blood cell analysis shows marked

lymphocytosis at birth and at later stages of life compared to adulthood. An increase in T and B lymphocytes occurs during the first weeks of life while NK cells decline sharply directly after birth [9, 10]. A high CD4/CD8 ratio in babies up to two years of age has been found compared to the values observed in normal adults, and this is mainly due to the large and expanding pool of naïve T CD4+ cells present during the first year of life [11, 12]. The proportion of activated T cells like CD3+CD25+ and CD3+HLA-DR+ are lower in newborns than in adults [13]. In contrast, the proportions of CD8+CD28+, CD8+CD38+, and CD4+CD38+ cells are higher in neonates [13, 14]. There are only a limited number of studies that have addressed the influence of milk-feeding practices [15-20] or type of labor or delivery [21-23] on peripheral lymphocyte subsets or the immunocompetence of lactating children.

Given the important role that the maturation of the immune system can play as a predisposing or protective factor to disease development later in life, we planned to assess the effect of type of delivery, gender, mother's antibiotic intake during pregnancy, mother's antibiotic administration during labor, milk-feeding practices, infections and antibiotic intake in the first 4 months of life, and rotavirus vaccine administration on the levels of lymphocyte subsets and their activation markers, and on the gut microbiota composition in infants at risk of developing CD.

MATERIALS AND METHODS

Subjects and information on environmental factors

This study was conducted in 4 month-old infants who are first-degree relatives of CD patients (at least one parent or sibling affected with CD). A total of 55 infants with these characteristics, born between October 2006 and the end of 2010 were recruited in the University Hospital La Paz and University Hospital Niño Jesús of Madrid. Questions about mode of delivery, infections, vaccinations, feeding practices, clinical symptoms, and medical treatments were answered by the parents at the infant's age of 1 and 4 months in clinical visits with the paediatric gastroenterologist. To facilitate the collection of this information, a diary was given to the mothers upon recruitment (before delivery). On the basis of these questionnaires, the 55 infants were divided according to the following variables that were selected as potential influential factors of immune system development and gut microbiota composition: type of delivery (vaginal/cesarean), mother's antibiotic intake during pregnancy (yes/no), mother's antibiotic administration during labor (yes/no), milk-feeding practices (breast-fed (BF) infants, including those which had been exclusively breast-fed until the age of 4 months, and formula-fed (FF) infants, including babies receiving milk formula, either alone or in alternation with breast milk at

the moment of analysis), infections in the first 4 months of life (yes/no), antibiotic intake in the first 4 months of life (yes/no), rotavirus vaccine administration (yes/no), and allergies or dermatitis suffering within the first 18 months of life (yes/no) (Table 1). The study was approved by the Ethic Committee of the University Hospital La Paz and the University Hospital Niño Jesús of Madrid, and written informed consent was obtained from the parents of infants included in the study. This group of infants was a subset of those recruited in 8 Spanish hospitals for the PROFICEL study [20].

Blood analyses

At the age of 4 months, peripheral blood samples were drawn from the infants and collected in BD Vacutainer™ tubes containing K3EDTA. Immediately after, 1 mL of blood was mixed with an equal volume (1 mL) of preservative solution (Streck Cell Preservative™ CE. Streck, USA) and sent to our laboratory at 4-8ºC within 2-6 days for a centralised processing and flow cytometry analysis. Blood extractions were always performed previous to the standardized 4 month vaccination in the Spanish vaccination schedule. Complete blood counts and differential counts were performed *in situ* at the corresponding enrolling centers by automated instrumentation. Blood samples were not taken if the infant presented an ongoing infection or had received a vaccination in the prior six weeks.

Flow cytometry analysis

Aliquots of blood mixed with the preservative-solution (150 µL) were incubated for 30 minutes at room temperature and in the dark with 20 µL of fluorochrome conjugated-monoclonal antibodies specific for CD3+ (CD3-APC), CD4+ (CD4-PerCP-Cy5.5), CD8+ (CD8- PerCP-Cy5.5), CD45RA+ (CD45RA-FITC), CD45RO+ (CD45RO-PE), CD25+ (CD25-FITC), HLA-DR+ (HLA-DR-FITC), CD38+ (CD38-PE), in quadruple immunostainings and 100 µL aliquots were incubated with the multitests CD3-FITC/CD16+56-PE/CD45-PerCP-Cy5.5/CD19-APC and CD3-FITC/CD8-PE/CD45-PerCP-Cy5.5/CD4-APC. All the monoclonal antibodies were purchased from Becton Dickinson (Sunnyvale, CA, USA). After incubation, samples were lysed with the BD FACSTM Lysing Solution (Becton Dickinson) following the manufacturer's protocol. The samples were analysed with FACSCalibur Flow Cytometer (four-color, dual-laser, Becton Dickinson). The lympho-gate was defined on the forward and side scatter patterns of lymphocytes. The analysis protocol gated on lymphocytes stained with PerCP and/or APC and the selected population was then analysed with the 2 remaining colours (FITC and PE) to obtain cell percentages expressing the specific antigens.

Cell subset counts were obtained by multiplying subset percentages by anchor marker counts, the later resulting by multiplying subset percentage by the absolute lymphocyte count.

Faecal sampling and DNA extractions

Stool samples were collected from 44 subjects at 4 months of age and frozen at −20°C immediately. Samples (1 g) were diluted 1:10 (w/v) in PBS (pH 7.2) and homogenized by thorough agitation in a vortex. Aliquots were used for DNA extraction using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA extractions from different pure cultures of reference strains were done following the same protocol.

Quantitative PCR (qPCR) analysis of faecal bacteria

The following bacterial groups were analysed: *Bifidobacterium* spp., *Lactobacillus* group, *Staphylococcus* spp.*, Bacteroides fragilis* group, *Clostridium coccoides-Eubacterium rectali* group, *Clostridium leptum* group*, Escherichia coli,* and the following *Bifidobacterium* spp.: *B. longum, B. breve, B. bifidum, B. adolescentis, B. catenulatum, B. angulatum, B. infantis, B. lactis,* and *B. dentium*. qPCR was used to quantify the different bacterial groups in faeces using genus-, group- and species-specific primers as previously described [24, 25]. Briefly, PCR amplification and detection were performed with an ABI PRISM 7000-PCR sequence detection system (Applied Biosystems, UK) using SYBR® Green PCR Master Mix (SuperArray Bioscience Corporation, USA). The bacterial concentration from each sample was calculated by comparing the Ct values obtained from standard curves of reference strains. Standard curves were created using serial 10-fold dilutions of pure culture DNA corresponding to 10^2 to 10^9 cells, as determined by microscopy counts after staining with 4',6-diamino-2-phenylindole in an epifluorescence microscope (Olympus BX51, Tokio, Japan).

Statistics

Associations of perinatal and early environmental factors (EEF) with lymphocyte subset values and bacteria counts were analysed using multiple linear regression models. In addition, logistic regression analyses were used to examine the effect of the environmental factors on the prevalence of colonization (colonized opposed to non-colonized). Previously, data with a non-normal distribution were transformed logarithmically to a normal distribution. Type of delivery, mother's antibiotic intake during pregnancy, mother's antibiotic administration during labor, milk-feeding practices, infections and antibiotic intake in the first 4 months of life, rotavirus vaccine administration, and allergy incidence were included in the models. To limit the chance of falsely

rejecting the null hypothesis (no association) as a result of multiple testing, a P-value lower than 0.010 was considered statistically significant in multiple linear regression analysis. Spearman's test was performed to assess possible correlations between exposures to the different individual perinatal and environmental factors and lymphocyte subsets, and also between the same factors and microbiota composition. Finally, correlations between lymphocyte subsets and faecal bacteria counts were equally performed. A P-value less than 0.050 was considered statistically significant.

RESULTS

Lymphocytes subsets

The associations of perinatal and EEF with the percentage and counts of the lymphocyte subsets under study, as determined in the linear regression analysis adjusted for gender, are presented in Table 2.

Formula-feeding was associated with higher absolute counts of total lymphocytes, T cells (CD3+), helper T cell (CD4+), CD4+CD38+, and CD4+CD28+ cells. T test analyses confirmed these results (P values ≤ 0.05 for these four subsets), and also showed that the percentage of CD4+CD25+ cells was higher in BF infants (11.1±2.9 vs. 9.3±3.3; P≤0.05) while CD4+CD38+ cell percentage was lower compared to FF infants (91.1±19.5 vs. 96.6±1.0; P≤0.05).

Infant's infections in the first 4 months of age were also associated with higher absolute counts of CD3+, CD4+, CD4+CD38+, and CD4+CD28+ cells (Table 3).

Regarding activation markers which are not constitutively expressed, cesarean delivery and rotavirus vaccine administration were associated with a lower percentage of activated T helper lymphocytes that express CD25+ (CD4+CD25+), in contrast, infants whose mothers used antibiotics during pregnancy were associated with a higher percentage of these cells. Infant's infections in the first 4 months of life were associated with higher absolute counts of CD4+CD25+ (Table 3).

Formula-feeding and infant's infections were associated with higher absolute counts of memory T helper lymphocytes (CD3+CD4+CD45RO+), and infant's antibiotic intake in the first 4 months was associated with lower absolute counts of these cells.

Infant's infections were associated with higher absolute counts of activated T helper lymphocytes HLA-DR+ (CD4+HLA-DR+) (Table 3).

Finally, infant's infections and antibiotic administration in mothers during labor were associated with higher percentage and absolute counts of NK cells (CD3-CD16+CD56+), and future allergy incidence was associated with a lower percentage of NK cells.

Spearman's test carried out to analyse the correlations between lymphocyte subsets and bacterial genera, groups and species, did not show significant results.

Gut microbiota composition

The composition of the fecal microbiota of 44 infants was analysed at 4 months of age (Table 4). The remaining 11 infants did not collect their stool samples.

The results of the linear regression analysis of bacterial counts with respect to environmental factors are shown in Table 4. Infants born by cesarean delivery showed associations with lower counts of *B. catenulatum*, and higher counts of *B. angulatum* (Table 5). Spearman correlations showed that infants born by cesarean delivery were correlated with lower counts of *B. catenulatum* (R=-0.514; P=0.010).

Infants whose mothers took antibiotic during pregnancy were associated with lower counts of *B. angulatum*.

Regarding milk-feeding practices, formula-feeding was associated with lower counts of *B. angulatum* in the infant.

Antibiotics intake in the first 4 months of life was associated with higher counts of *Bacteroides fragilis* group and *B. angulatum*, and lower counts of *B. longum* (Table 5). Spearman tests also showed significant correlations between infant's antibiotic intake and higher counts of *Bacteroides fragilis* group (R=0.433; P=0.019), higher *B. angulatum* prevalence (R=0.339; P=0.032), and lower counts of *Bifidobacterium* spp. (R=-0.323; P=0.042) and *B longum* (R=-0.443; P=0.004).

Allergy and dermatitis incidence at follow-up was associated with lower counts of *B. angulatum* at 4 months.

Rotavirus vaccine administration was associated with lower counts of *B. angulatum* and was also significantly correlated with a lower prevalence of *Bacteroides fragilis* group (R=-0.396; P=0.017). The rest of analysed bacteria did not show significant results for any of the EEF evaluated.

Finally, the logistic regression analysis did not show significant associations between the different EEF and the prevalence of bacterial colonization.

DISCUSSION

Several EEF could be involved in immune system development and intestinal colonization in early life. It is well known that gut microbiota interacts with the gut-associated lymphoid tissue

(GALT) and could thereafter modulate the systemic immune system. In this study, we assessed the associations between different EEF, peripheral lymphocyte subsets, and microbiota composition in fifty-five 4 month-old infants with at least one first-degree relative suffering from CD.

A widespread increase in the number of lymphocytes was associated with formula feeding in the studied population. The increase in lymphocytes involved mainly the T helper subsets, the overall subset (CD4+CD38+ and CD4+CD28+) as well as specifically the memory helper T cells. Similarly, an increase in the CD3+ and CD4+ lymphocyte counts were also associated with infant´s infections, although in these cases, in addition to increased memory cell counts, also an increase in activated subsets expressing CD4+CD25+, and CD4+HLA-DR+ were associated with infection processes. Similar to our results, a study carried out with infants (0 to 6 months of age) of healthy mothers and uninfected infants of HIV-infected mothers, in which CD4+ lymphocyte phenotypes were examined, found that the proportions of activated (CD4+ HLA-DR+ CD38+) and memory (CD4+ CD45RA- RO+) lymphocytes were increased in uninfected infants of HIV-infected mothers compared to infants of uninfected mothers. Therefore, it was concluded that the lymphocytes of some HIV-exposed, uninfected infants, had been stimulated by antigen at early age and the cells principally implicated were helper T cells [26]. Early infections would reasonably result in a higher proportion of memory and activated T helper cells as shown in our infants, however, regarding the association of formula-feeding with higher memory helper T cells, no similar study has been found in the literature to compare with, although an increase in T cells, particularly in T helper cells [15, 17], and a decrease in NK cells [17] have been reported for FF infants compared with BF infants at 6 months of age. Surprisingly, on the contrary to formula feeding and infant´s infections, lower numbers of memory CD4+ cells were associated with the intake of antibiotics in the first 4mo., which suggest that taking antibiotics to halt the infections, alters the balance of immune cells acquired when infection is being eliminated by the body's own defense mechanisms. *In vivo* studies indicate that shortening the length of *Listeria monocytogenes* infection by antibiotic treatment can affect CD4+ T cell activation and development of memory T cells, but this effect is dependent on infectious dose, suggesting that overall antigen levels may play a role [27].

The percentage of CD4+CD25+ cells was stimulated by vaginal delivery, antibiotics use by mothers during pregnancy, and breast-feeding, but in contrast, a lower percentage was associated with infants vaccinated against rotavirus. A recent study [28] that compared the patterns of gene expression in peripheral blood mononuclear cells (PBMCs) from children with rotavirus diarrhea and healthy children showed that the first group had increased expression of genes involved in B-cell differentiation, maturation, activation and survival, but lower levels of mRNA for

genes involved in the various stages of T-cell development. Importantly, this study also demonstrated a reduction in the total lymphocyte population and in the proportions of CD4+ and CD8+ T cells in PBMCs, suggesting that rotavirus alters T-cell homeostasis. According to our results, the rotavirus vaccine also influences the balance between T and B lymphocytes, as we have found by T test analysis that B cell percentage is higher in vaccinated infants (data not shown). On the other hand, previous results by our group in a bigger population with similar characteristics showed a higher percentage of CD4+CD25+ cells in BF infants than in FF infants [20], which is in agreement with the results in the current study.

According to the hygiene hypothesis, early infections caused by certain microorganisms are suspected to accelerate the Th1 maturation in children and to protect against future allergic disease [29]. Our study showed that suffering infections during the first 4 months of life was associated with higher NK cell numbers. A similar result was found in a study comparing septic and healthy infants and children that described increased NK cells in the infants even after recovery from sepsis [30]. Moreover the development of allergy or dermatitis within the first 18 months of life was associated with a lower percentage of NK cells at 4 months. Accordingly, studies in newborns at risk for allergy [31], infants with multiple allergy food [32], and children with atopic dermatitis [33] showed a decreased percentage [32, 33] and counts [31] of NK cells. Our data suggest that infections suffered during the first 4 months of life might be protective, in the long term, for allergy and dermatitis development through the effect of infection on NK cells. However, no correlation was found between infection during the first 4 months and allergy incidence in the next months. Although, we have not found this correlation, the study by Han et al. [34] support this last hypothesis. They suggest that NK cells may play an important role in infection-mediated inhibition of allergic responses. They studied the effect of chlamydial infection on the development of allergic responses induced by ovalbumin (OVA) and the involvement of NK cells in this process using a mouse model of airway inflammation. They found that the adoptive transfer of NK cells that were isolated from infected mice and injected to a syngeneic naïve mice before OVA sensitization, produced a decrease of circulating and infiltrating eosinophils in the lung and inhibited the production of Th2 cytokines (IL-4 and IL-5) of spleen cells after sensitization.

Infants born by cesarean delivery are deprived of contact with their mother's intestinal and vaginal microbiota. In this infants, the acquisition of bacteroides , bifidobacteria, and *E. coli* has been shown to be delayed [8]. Accordingly, we found that cesarean delivery was associated with lower counts of *B. catenulatum*. Similar results were also found in the KOALA study, which showed that infants born by cesarean section had lower numbers of bifidobacteria and

bacteroides [35]. On the other hand, a retrospective, multicenter, case-control study that included 1950 children found a significantly enhanced likelihood of being born by cesarean delivery in children with CD compared with control subjects [36]. The authors hypothesized that the influence of cesarean delivery on the postnatal establishment of the intestinal microbiota might primarily affect the neonatal period and therefore enhance the intestinal epithelial permeability to gluten, leading to an aberrant stimulation of the mucosal immune system. This matter will possibly be elucidated after adequate length of follow-up is achieved for the cohort of the current study.

Oral use of antibiotics by the infant during the first 4 months also showed several associations with faecal microbiota composition, such as lower counts of *B. longum* and *Bifidobacterium* spp. The KOALA study also found lower counts of bifidobacteria in infants who received oral antibiotic therapy in their first 1 month [36]. Finally, counts of *B. angulatum* appear to be associated with many of the different environmental factors studied (type of delivery, mother's antibiotic intake during pregnancy, milk-feeding practices, infections and antibiotic intake in the first 4 months of life, and rotavirus vaccine administration) which suggest that this might be a bacterial species more susceptible to fluctuations that others in its group.

One important point to consider about this study is the fact that it was performed in a population selected by the familial risk to suffer CD, which means that our results cannot be extrapolated to the general population. In this sense, we know that the frequency of infants with HLA haplotype different from DQ-2 in the group studied is significantly lower that the proportion in the general population [20]. On the other hand, we have observed previously in a similar population that no interaction effect exists between milk-feeding practices and HLA-genotype on lymphocyte subsets in these infants [20]. However, we cannot rule out the selection bias effect and therefore the importance of the influence of EEF on lymphocyte subsets and microbiota development would only be known in the future, when we are able to assess if the associations found here impact on CD development in this infant cohort.

In conclusion, infant´s infections and antibiotic intake in the first 4 months of life are the EEF more strongly and/or frequently associated to lymphocyte subpopulations and microbiota composition, respectively in infants at risk of CD, but other factors such as milk-feeding practices, type of delivery, prenatal exposure to antibiotics, and rotavirus vaccine were also associated. The balance between the effects on lymphocyte subpopulations and microbiota composition of all these pre- and post-natal factors might modulate and define the risk for the future development of immune-related diseases such as CD. Thus, longer follow up of the entire PROFICEL cohort is necessary to assess the combined effect of environmental factors on that risk.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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A continuación se presentan las tablas del presente artículo, ya que el artículo no se incluye en el ANEXO I por no estar aun publicado.

Table 1. Demographic data of the study infants

		Total subjects (n=55)
Gender		
	Male	26
	Female	29
Delivery		
	Vaginal	43
	Cesarean	12
Antibiotics during pregnancy		
	Yes	15
	No	39
Antibiotics during labor		
	Yes	8
	No	46
Milk-feeding		
	Breastfeeding	24
	Formula-feeding	31
Infections in first 4 mo.		
	Yes	33
	No	21
Antibiotics in first 4 mo.		
	Yes	12
	No	42
Allergy or dermatitis		
	Yes	18
	No	29
Rotavirus vaccine		
	Yes	17
	No	32

Table 2. Linear regression coefficients for lymphocyte subset percentages and counts, with respect to environmental factors in multivariate analyses (n=55)

		Type of delivery (vaginal/cesarean)*	Antibiotics pregnancy (no/yes)	Antibiotics labor (no/yes)	Milk-feeding (Breastfeeding/Formula-feeding)	Infant's infections (no/yes)	Infant's Atbs. (no/yes)	Allergy/dermatitis (no/yes)	Rotavirus vaccine (no/yes)
		Coefficient (P)							
Lymphocytes	cel./µL	NS	NS	NS	0.466 (0.010)	NS	NS	NS	NS
CD3+	%	NS	NS	NS	NS	NS	NS	NS	NS
	cel./µL				0.465 (0.010)	0.608 (0.010)			
CD4+	%	NS	NS	NS	NS	NS	NS	NS	NS
	cel./µL				0.475 (0.010)	0.583 (0.008)			
CD4+CD38+	%	NS	NS	NS	NS	NS	NS	NS	NS
	cel./µL				0.527 (0.003)	0.640 (0.002)			
CD4+CD28+	%	NS	NS	-0.447 (0.004)	NS	NS	NS	NS	NS
	cel./µL			NS	0.477 (0.010)	0.576 (0.009)			
CD25+	%	NS	NS	NS	NS	NS	NS	NS	-0.465 (0.005)
	cel./µL								-0.402 (0.010)
CD4+CD25+	%	-0.392 (0.007)	0.378 (0.008)	NS	NS	NS	NS	NS	-0.476 (0.002)
	cel./µL	NS	NS			0.499 (0.009)			NS
CD3+CD4+CD45RO+	%	NS	NS	NS	NS	NS	NS	NS	NS
	cel./µL				0.470 (0.010)	0.601 (0.004)	-0.513 (0.009)		
CD4+HLA-DR+	%	NS	NS	NS	NS	NS	NS	NS	NS
	cel./µL					0.563 (0.010)			
CD3-CD16+CD56+	%	NS	NS	0.417(0.004)	NS	0.488 (0.005)	NS	-0.338 (0.010)	NS
	cel./µL			0.504 (0.001)		0.639 (0.000)		NS	

Results for lymphocyte subsets with no statistically significant results for any of the environmental factors are not presented. Coefficients are regression coefficients of association between environmental factors and percentages and counts of lymphocyte subsets; models included the following independent variables: Type of delivery, mother's antibiotic intake during pregnancy, mother's antibiotic administration during labor, milk-feeding practices, infections and antibiotic intake in the first 4 months of life, allergy/dermatitis incidence and rotavirus vaccine administration. All models were adjusted for gender.*The sign of the association refers to the category appearing in the second place. Statistically significant results (P≤0,01). NS: Not statistically significant results.

Table 3. Medians and quartiles of lymphocyte subsets percentages and absolute counts of infants 4 months of age (n=55)

Infections in first 4 months				
	Yes		No	
No.	34		21	
%	Mean	Q1-Q3	Mean	Q1-Q3
CD3+	64,77	59,83-71,45	62,75	59,35-67,41
CD4+	48,98	42,93-54,70	47,79	43,09-51,94
CD8+	12,72	11,85-18,23	14,84	12,70-21,38
Ratio CD4/CD8	3,66	2,55-4,46	2,99	2,22-3,96
CD4+CD28+	100	99,77-100	100	99,85-100
CD8+CD28+	95,18	92,46-97,21	94,98	89,09-97,20
CD4+CD38+	96,30	95,20-97,25	96,37	95,27-97,30
CD8+CD38+	95,55	93,55-97,48	94,78	90,59-97,14
CD4+HLA-DR+	3,35	2,39-4,92	2,68	2,06-3,39
CD4+HLA-DR+CD38+	2,71	1,85-4,05	2,38	1,54-2,86
CD8+HLA-DR+	3.60	2,60-6,65	3,35	2,03-5,14
CD8+HLA-DR+CD38+	3,60	2,35-6,33	2,98	1,63-4,75
CD25+	6,91	5,36-8,60	6,09	5,00-7,10
CD4+CD25+	10,84	8,62-11,85	9,58	7,57-11,34
CD8+CD25+	1,49	0,94-2,15	1,57	0,69-1,75
CD3+CD45RO+	11,52	8,97-13,94	11,00	8,15-14,22
CD4+CD45RA+	89,18	84,37-90,53	88,37	83,48-90,96
CD3+CD4+CD45RO+	8,34	6,04-10,23	8,11	5,20-10,77
CD8+CD45RA+	90,74	85,37-92,32	92,33	86,14-96,55
CD3+CD8+CD45RO+	1,87	1,42-3,18	1,80	1,00-3,41
CD3-CD16+CD56+	6,64	4,73-8,87	5,12	3,85-5,96
CD19+	23,71	17,91-27,55	26,65	20,50-29,97
Counts (cel./µL)	Mean	Q1-Q3	Mean	Q1-Q3
Lymphocytes	5520	4910-7200	5270	4490-6040
CD3+	3720	3068-4576	3209	2923-3758
CD4+	2913	2062-3466	2411	2230-2970
CD8+	844	635-1074	794	595-1040
CD4+CD28+	2888	2062-3466	2411	2230-2969
CD8+CD28+	797	598-1038	750	571-839
CD4+CD38+	2693	1947-3334	2262	2138-2608
CD8+CD38+	803	624-1043	706	553-910
CD4+HLA-DR+	113	67-145	70	48-85
CD4+HLA-DR+CD38+	85	54-120	57	36-66
CD8+HLA-DR+	38	21-60	22	13-32
CD8+HLA-DR+CD38+	36	19-57	19	10-31
CD25+	418	322-535	324	268-366
CD4+CD25+	305	231-405	202	235-252
CD8+CD25+	10	7-24	9	5-16
CD3+CD45RO+	440	319-528	384	283-490
CD4+CD45RA+	2446	1817-3081	2024	1840-2651
CD3+CD4+CD45RO+	326	229-395	240	205-375
CD8+CD45RA+	718	550-997	726	553-903
CD3+CD8+CD45RO+	80	48-136	48	33-120
CD3-CD16+CD56+	416	240-634	263	195-375
CD19+	1361	912-1863	1534	962-1769

Table 4. Linear regression coefficients for bacterial counts with respect to environmental factors in multivariate analysis (n=44)

	Type of delivery (vaginal/cesarean)*	Antibiotics pregnancy (no/yes)	Antibiotics 4 mo (no/yes)	Milk-feeding (Breastfeeding/Formula-feeding)	Allergy/dermatitis (no/yes)	Rotavirus vaccine (no/yes)
	Coefficient (P)	Coefficient (P)	Coefficient (P)	Coefficient (P)	Coefficient (P)	Coefficient (P)
Bacteroides fragilis	NS	NS	0.585 (0.033)	NS	NS	NS
B. longum	NS	NS	-0.513 (0.011)	NS	NS	NS
B. catenulatum	-0.743 (0.006)	NS	NS	NS	NS	NS
B. angulatum	0.719 (0.021)	-1.351 (0.015)	1.321 (0.020)	-1.585 (0.013)	-1.170 (0.021)	-0.838 (0.017)

Results for bacteria with no statistically significant results for any of the environmental factors are not presented. Regression coefficients of association between environmental factors and bacterial counts are presented in the first column for each independent variable; models included the following independent variables: Type of delivery, mother's antibiotic intake during pregnancy, mother's antibiotic administration during labor, milk-feeding practices, infections and antibiotic intake in the first 4 months of life, allergy/dermatitis incidence and rotavirus vaccine administration. All models were adjusted for gender. *The sign of the association refers to the category appearing in the second place. Statistically significant results (P≤0,05). NS: Not statistically significant results.

Table 5. Prevalence of colonization (%), medians, and quartiles (Q1-Q3) of bacteria counts (log_{10} CFU/g faeces)

	Antibiotics in first 4 months						Type of delivery					
	Yes			No			Vaginal			Cesarean		
No.	11			33			34			10		
	%	Median	Q1-Q3	%	Median	Q1-Q3	%	Median	Q1-Q3	%	Median	Q1-Q3
Bifidobacteria spp.	100	6,91	6,42-7,23	100	7,54	6,83-7,97	100	7,40	6,72-7,96	100	7,20	6,84-7,67
Bacteroides fragilis	70	7,74	6,36-8,57	73	5,86	3,95-6,92	81	6,20	4,17-7,47	44	6,14	4,40-7,84
Staphylococcus spp.	70	5,70	4,25-6,72	57	5,67	4,70-6,26	61	5,38	4,70-5,97	56	6,34	5,09-6,48
Clostridium coccoides	100	6,31	5,10-7,67	100	5,66	4,76-6,96	100	5,98	5,36-7,29	100	5,09	4,16-6,75
Clostridium leptum	80	5,29	4,41-6,01	70	5,00	4,41-5,31	64	5,11	4,44-5,89	100	4,94	4,21-5,07
Lactobacillus	100	6,66	5,97-8,24	100	6,57	5,84-7,91	100	6,55	5,79-7,89	100	7,34	6,61-8,14
Escherichia coli	100	8,06	6,73-8,73	93	7,05	5,70-8,00	93	7,65	5,82-8,12	100	7,22	5,03-8,25
Bifidobacterium longum	100	5,14	4,53-6,00	100	6,55	6,04-7,21	100	6,29	5,27-7,09	100	6,54	4,50-6,76
Bifidobacterium breve	100	6,74	4,55-7,27	90	6,05	4,45-7,35	93	6,05	4,52-7,03	89	6,53	4,56-7,35
Bifidobacterium bifidum	100	5,55	3,99-6,81	87	5,08	4,53-6,36	87	5,51	4,42-6,54	100	4,61	4,22-5,35
Bifidobacterium adolescentis	30	6,00	5,60- -	20	5,37	4,38-6,16	19	6,00	5,27-6,16	33	5,30	4,41- -
Bifidobacterium catenulatum	70	6,24	3,75-7,36	57	5,22	4,44-6,94	58	6,24	4,80-7,25	67	4,44	3,64-4,65
Bifidobacterium angulatum	60	5,20	4,53-5,40	23	5,11	4,19-5,41	35	5,19	4,39-5,39	22	4,80	4,19- -
Bifidobacterium infantis	10	5,42	5,42-5,42	43	5,37	5,10-7,97	35	5,23	5,09-6,90	33	6,94	6,74- -
Bifidobacterium lactis	40	5,30	4,30-5,66	23	5,18	4,19-5,74	26	5,30	4,92-5,74	33	4,19	4,00- -
Bifidobacterium dentium	20	5,27	5,27- -	3	4,16	4,16-4,16	6	5,27	5,18- -	10	4,16	4,16-4,16

2. Efecto inmunoestimulador *in vitro* de diferentes cepas bacterianas intestinales características de niños alimentados con leche materna y leche de fórmula sobre CMNs, y en sistemas de co-cultivo CMNs/células Caco-2

I. Immunostimulatory effect of faecal *Bifidobacterium* species of breast-fed and formula-fed infants in a peripheral blood mononuclear cell/Caco-2 co-culture system [148].

I. Immunostimulatory effect of faecal *Bifidobacterium* species of breast-fed and formula-fed infants in a peripheral blood mononuclear cell/Caco-2 co-culture system [148].

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(ver ANEXO I)

RESUMEN

Se cree que *Bifidobacterium* spp. típicas de la microbiota intestinal humana influyen en el balance de las respuestas inmunológicas de la mucosa intestinal.

El objetivo del presente estudio fue investigar el efecto de diferentes especies de bifodobacterias y sus mezclas en experimentos *in vitro* con células mononucleares de sangre periférica (CMNs) y células Caco-2.

Se usaron las bacterias *Bifidobacterium adolescentis*, *B. angulatum*, *B. breve*, *B. catenulatum*, *B. infantis*, *B. longum,* y dos combinaciones de estas bifidobacterias simulando la composición de especies encontradas en muestras fecales de niños alimentados con leche materna (LM) y leche de fórmula (LF). Se midieron los niveles de varias citoquinas segregadas por las CMNs y por el co-cultivo Caco-2/CMNs mediante estimulación con las bifidobacterias.

B. catenulatum y *B. breve* fueron las más fuertes inductoras de producción de IFN-γ por la estimulación directa de CMNs. *B. longum* fue la mayor inductora de IL-10 y la menor de TNF-α. En el sistema de co-cultivo CMNs/Caco-2, *B.breve* fue la mayor inductora de IL-8 por parte de las células Caco-2, y significativamente diferente de *B. infantis*, *B. adolescentis*, y la mezcla LF ($P<0.05$). El IFN-γ producido por las CMNs estimuladas con la mezcla LM (que contiene 22% de *B. breve*, comparado con un 7% en la mezcla LF) fue significativamente mayor comparado con *B. adolescentis*, *B. infantis* y *B. longum*. También, *B. adolescentis* inhibió la producción de IFN-γ comparado con la mezcla LF y *B. longum*.

La proporción de las diferentes cepas de bifidobacterias parece ser un importante determinante del balance de citoquinas en el ambiente intestinal simulado en el estudio. *B. breve* y la combinación de las especies de *Bifidobacterium* típicamente encontradas en la microbiota de niños alimentados con leche materna mostraron los efectos más significativos.

ABSTRACT

Bifidobacterium spp. typical of the human intestinal microbiota are believed to influence the balance of immune responses in the intestinal mucosa.

The aim of the present study was to investigate the effect of different bifidobacterial species and their mixtures in *in vitro* experiments with peripheral blood mononuclear cells (PBMC) and Caco-2 cells. *Bifidobacterium adolescentis*, *B. angulatum*, *B. breve*, *B. catenulatum*, *B. infantis*, *B. longum* and two combinations of these bifidobacteria simulating the species composition found in faecal samples from breast-fed (BF) and formula-fed (FF) infants were used. The levels of several cytokines were measured by direct stimulation of PBMC and by stimulation of a Caco-2/PBMC co-culture with bifidobacteria.

B. catenulatum and *B. breve* were the strongest enhancers of interferon-γ (IFN-γ) production by direct stimulation of PBMC. *B. longum* was the highest inducer of IL-10 and the lowest TNF-α stimulus. In the Caco-2/PBMC system, *B. breve* was the highest inducer of IL-8 production by Caco-2 cells, significantly different from *B. infantis*, *B. adolescentis* and the FF mixture ($P<0.05$). IFN-γ produced by PBMC stimulated with the BF mixture (containing 22% *B. breve*, compared with 7% in the FF mixture) was significantly higher compared with *B. adolescentis*, *B. infantis* and *B. longum*. *B. adolescentis* also inhibited IFN-γ production compared with the FF mixture and *B. longum*.

The proportion of different *Bifidobacterium* strains seems to be an important determinant of the cytokine balance in the simulated intestinal environment studied. *B. breve* and the combination of the *Bifidobacterium* species typically found in the microbiota of BF infants have shown the most significant effects.

Key words: *Bifidobacterium* spp.; Caco-2 cells; Peripheral blood mononuclear cells; Cytokines; Breast-feeding; Formula feeding; Infant's microbiota

Abbreviations:

BF	Breast-fed
FF	Formula-fed
IEC	intestinal epithelial cells
IFN-γ	interferon-γ
PBMC	peripheral blood mononuclear cells

INTRODUCTION

The intestinal microbiota plays a pivotal role in human health by preventing pathogen colonisation, and shaping and maintaining normal mucosal immunity (1). To preserve this beneficial relationship, the immune system should remain hyporesponsive to commensal bacteria (mucosal tolerance) (2,3), but at the same time, it has to combat pathogenic bacteria (3). The breakdown of the delicate balance of the intestinal immune responses causes the development of disease states with bowel inflammation (3). In this context, intestinal epithelial cells (IEC) play an important role in immune homeostasis (4,5). IEC are thought to contribute to immunomodulation of mucosal leucocytes by at least two different mechanisms (6), by acting as a physical barrier between gut luminal content (including bacteria) and the underlying immune cells, and by transmitting signals coming from the intestinal content and microbiota to the resident mucosal immune system(4). IEC secrete many mediators involved in protective responses against potentially pathogenic organisms, such as defensins, mucins, chemokines and cytokines (5).

Bifidobacteria, which are important components of the human intestinal microbiota particularly of breast-fed (BF) infants (7), have shown the capacity to modulate cytokine production by IEC, monocyte-derived dendritic cells and peripheral blood mononuclear cells (PBMC) in *in vitro* experiments (1,8,9). In addition, the differences observed in the composition of bifidobacterial species of the intestinal microbiota of BF and formula-fed (FF) infants have been suggested to influence the incidence of immune-mediated diseases (10,11). These findings have led to the proposal to use some *Bifidobacterium* strains as potential probiotics in the prevention and treatment of pathologies with underlying immune alterations, such as inflammatory bowel diseases, allergy and coeliac disease (12-14).

Following all of the aforementioned facts and hypothesis, the objective of the present study was to investigate the effect of strains of different bifidobacterial species (*Bifidobacterium adolescentis, B. angulatum, B. breve, B. catenulatum, B. infantis and B. longum*) and their mixtures, corresponding to the typical microbiota present in the faeces from BF and FF children, on the modulation of cytokine production by IEC and PBMC in an in vitro co-culture system, simulating the intestinal environment.

MATERIALS AND METHODS

Bacteria

The following strains of six different *Bifidobacterium* species were individually evaluated: *B. adolescentis* ATCC 15703; *B. angulatum* ATCC 27535; *B. breve* ATCC 15700; *B. catenulatum* LMG 11043; *B. longum biovar infantis* LMG 11046T; *B. longum biovar longum* ATCC 15707. In addition, two combinations of these bifidobacteria were also used to simulate the percentage of each species in the microbiota from BF and FF infants (10). The BF mixture included *B. infantis* (59.0 %), *B. breve* (21.6 %), *B. longum* (13.5 %), *B. catenulatum* (3.5 %), *B. angulatum* (1.8 %) and *B. adolescentis* (0.6 %); the FF mixture included *B. infantis* (62.1 %), *B. catenulatum* (14.8 %), *B. longum* (10.9 %), *B. breve* (7.2 %) and *B. adolescentis* (5.0 %) (no *B. angulatum*).

Bifidobacteria were grown routinely in de Man Rogosa and Sharpe agar (Scharlau Chemie SA, Barcelona, Spain) with 0.05% cysteine broth and incubated at 37ºC under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK) for 22 h. Cells were harvested by centrifugation (6000 *g* for 15 min) until the stationary growth phase, washed two times in PBS (130mM-NaCl, 10mM-sodium phosphate, pH 7.4, and resuspended in PBS containing 20% glycerol). Aliquots of these suspensions were frozen in liquid N_2 and stored at -80ºC until used. The number of live cells after storage was determined by colony-forming unit counting on de Man Rogosa and Sharpe-cysteine after 48 h incubation in optimal conditions. For all strains tested, >90% of cells were alive upon thawing. For every new experiment, one fresh aliquot was thawed to avoid variability in cultures between the experiments.

Leucocyte isolation and bacterial stimulation of peripheral blood mononuclear cells

Human PBMC from seven healthy volunteers were isolated from heparinised blood samples using standard Ficoll gradient centrifugation (lymphocyte isolation solution; Rafer, Zaragoza, Spain). The isolated PBMC were washed twice with Roswell Park Memorial Institute 1640 medium (Bio-Whittaker, Verviers, Belgium) and suspended in the same medium, supplemented with heat-inactivated fetal bovine serum (100 ml/l; Bio-Whittaker), after decomplementation, and containing 1% penicillin-streptomycin (5000 IU/ml (3mg/ml), 5000 mg/ml; Bio-Whittaker). The PBMC suspension was adjusted to $2x10^6$ cells/ml, and $1x10^6$ cells were used per well in all experiments.

Live bacterial cell suspensions of each individual *Bifidobacterium* strain or the combinations representing the faecal microbiota composition of BF and FF infants were washed in the culture medium and incubated at a final concentration of 10^7 colony-forming units/ml with PBMC

(proportion bacteria:PBMC, 10:1)(15,16) during 48 h (5% CO_2 and 37ºC). The supernatant was collected, centrifuged and frozen in aliquots at -80ºC until cytokine analysis.

Co-culture of Caco-2/peripheral blood mononuclear cells and bacterial stimulation

The colonic adenocarcinoma cell line Caco-2 (ECACC no. 86 010 202, Salisbury, Wiltshire, UK) was cultured at 37ºC and 5% CO_2 in Eagle's minimal essential medium (Bio-Whittaker) supplemented with 10% fetal bovine serum (Bio-Whittaker), 1% non-essential amino acid solution (Bio-Whittaker), 1% L-glutamine (Bio-Whittaker) and 1% penicillin-streptomycin (Bio-Whittaker). Caco-2 cells were seeded at a density of $8x10^4$ cells/well in standard twenty-four- well culture plates, and at $4x10^4$ cells/well on 12mm inserts in twenty-four-well cell culture plate assemblies (Millipore, Madrid, Spain) with a semipermeable polyethylene terephthalate membrane (1 mm in pore size). During cell growth and differentiation, the medium was changed every 2 or 3 d. Once the cells were confluent and differentiated, the experiments were performed 10–11 d after seeding. Confluence was followed by microscopic visualization and transepithelial resistance measurements (Millicell ERS Ohmmeter; Millipore).

Co-cultures of the bifidobacteria with Caco-2 cells and PBMC from healthy donors were performed in seven different experiments. To that end, a transwell cell culture system was used as described earlier. Caco-2 monolayers were challenged by apical addition of $2x10^6$ colony-forming units/insert of a *Bifidobacterium* strain or a combination of strains corresponding to the species composition in the faecal samples from BF and FF infants. The PBMC suspension (500ml) was added at a concentration of $2x10^6$ cells/ml in the basal compartment of the culture well for a 12 h incubation. Thereafter, further 36 h incubation was allowed after disassembly of the system. In order to measure the cytokine production by the sensitised Caco-2 and PBMC separately, the basolateral compartment of Caco-2 cells was replenished with a fresh culture medium. After the incubation period, culture media, from both the separated PBMC and Caco-2 cell plates, were collected and frozen in aliquots at -80ºC. The PBMC supernatant was centrifuged before freezing to avoid the presence of cells in aliquots.

In two different wells, two more conditions, which served as a control of the Caco-2 cell conditioning by the underlying PBMC, were carried out: the BF and FF mixtures were added to the Caco-2 monolayers in transwells with no PBMC in the basal compartment.

Cytokine quantification in culture supernatants

TNF-α, IL-1β, IL-10, IL-8 and IL-6 cytokines were measured in the basolateral medium with Caco-2 cells, and TNF-α, interferon-γ (IFN-γ), IL-6, IL-10, IL-2 and IL-4 were measured in the PBMC supernatant. All cytokine measurements were performed using a cytometric bead array system (Inflammation Kit and either a Th1/Th2 kit or a Flex set; BD Biosciences, San Agustín de Guadalix, Madrid, Spain), according to the manufacturer's protocols, and analysed by flow cytometry (FACScalibur; BD Biosciences). Data were analysed using Cellquest software (BD Biosciences). The cytometric bead array limit of detection for each cytokine was as follows: IFN-γ, 7.1 pg/ml; TNF-α, 2.8 pg/ml; IL-10, 0.13 pg/ml; IL-6, 1.6 pg/ml; IL-8, 1.2 pg/ml; IL-4, 2.6 pg/ml; IL-2, 2.6 pg/ml; IL-1b, 7.2 pg/ml. IFN-γ was also measured with high-sensitivity Immunoassay xMAP Technology (Millipore) in a Luminex 100 equipment, with a sensitivity of 0.29 pg/ml.

Statistical analyses

Statistical analyses were performed using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). To establish the homogeneity of variances and the distribution of the data, the Levene test was run. As a result of the non-normal distribution of the data and the non-homogeneity of the variances, the Mann–Whitney U test was used to assess the effect of every experimental condition compared with the other conditions. Data are expressed as medians and quartiles. Significant differences were established at $P<0.05$. Correlations between different bacterial stimulatory conditions were analysed by Spearman's correlation test and considered significant at a P level <0.05.

Ethical approval

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects/patients were approved by the Ethics Committees of the Hospital Puerta de Hierro (Madrid, Spain) and CSIC organisation. Written informed consent was obtained from all subjects/patients.

RESULTS

Cytokine production by peripheral blood mononuclear cells cultured with bifidobacteria

In order to determine the immunological effect of bifidobacteria on PBMC, the production of IFN-γ, TNF-α, IL-10, IL-6, IL-4 and IL-2 was measured in the supernatants of PBMC cultured in direct contact with the different *Bifidobacterium* strains (individually or mixed). Among all cytokines analysed, only IL-2 was not stimulated (Fig. 1(A)), with levels *below* 20 pg/ml (except for the positive control with phytohaemaglutinin; data not shown). All the other cytokines were significantly stimulated by all *Bifidobacterium* species and their mixtures (compared with the control with only medium).

Regarding IFN-γ production (Fig. 1(B)), *B. catenulatum* and *B. breve* were the strongest enhancers, followed by the FF and BF mixtures (no significant differences were found between the two mixtures). *B. catenulatum* induced a higher IFN-γ production than all the other stimuli (except for *B. breve*). *B. breve* induced a higher IFN-γ production than *B. adolescentis*, *B. angulatum* and *B. infantis*, but similar to that induced by *B. catenulatum*, *B. longum* and the mixtures. The total percentage of *B. catenulatum* and *B. breve* was similar in the FF and BF mixture (22.05 and 25.12%, respectively). This might explain why the levels of IFN-γ produced by PBMC stimulated with the FF and BF mixtures were not statistically different.

B. longum and *B. catenulatum* induced the highest IL-10 production by PBMC, showing significant differences in IL-10 production in the presence of *B. infantis* and the BF mixture (Fig. 1(C)). IL-10 production induced by *B. longum* was also significantly higher than that induced by *B. angulatum*, *B. breve* and the FF mixture. The percentages of *B. longum* in the FF and BF mixtures were very similar (10.87 *v.* 13.52 %), but *B. catenulatum* was approximately four times higher in the FF mixture than in the BF mixture (14.84 *v.* 3.50 %). The low proportion of *B. catenulatum* and *B. adolescentis*, together with the high proportion of *B. infantis* and *B. breve* in the BF mixture, might explain the significantly lower production of IL-10 induced by the BF mixture compared with that induced by *B. adolescentis*, *B. catenulatum* and *B. longum* individually (Fig. 1(C)). Regarding IL-4, *B. catenulatum* also induced a significantly higher production than *B. adolescentis* and *B. infantis* (Fig. 1(D)).

All *Bifidobacterium* strains stimulated PBMC to produce very high levels of IL-6, over 4000 pg/ml (Fig. 1(E)). *B. adolescentis* induced the highest IL-6 production, significantly higher than *B. angulatum*, *B. breve* and *B. infantis* (P=0.029 in every case). *B. infantis* induced the lowest effect among the assayed strains on cytokine production, not only for IL-6, but also for IFN-γ, IL-10 and IL-4.

With the exception of *B. adolescentis*, all *Bifidobacterium* strains also stimulated PBMC to produce very high levels of TNF-α (Fig. 1(F)). A significantly higher TNF-α production was induced by *B. angulatum* and *B. catenulatum* compared with *B. adolescentis*, *B. breve* and *B. longum*. While *B. longum* and *B. adolescentis* induced a high production of IL-10, they both mildly induced TNF-α production (Fig. 1(C) and (F)). On the other hand, while *B. infantis* and *B. angulatum* induced a mild production of IL-10, they both highly induced TNF-α production (Fig. 1(C) and (F)).

Cytokine production by peripheral blood mononuclear cells in co-culture with Caco-2 cells and bifidobacteria

To analyse cytokine production by PBMC conditioned by previous co-culture with Caco-2 cells stimulated with bifidobacteria, IFN-γ, TNF-α, IL-10, IL-6, IL-4 and IL-2 were measured in PBMC supernatants. IL-2 and IL-4 were not detectable, and TNF-α was also below the limit or approaching the limit of detection (data not shown). No significant differences in IL-10 and IL-6 production were found, either between different bifidobacteria alone or in mixtures (Fig. 2(A) and (B)).

The production of IFN-γ by PBMC was low in this system (range 1–93 pg/ml and under the detection limit in two of the seven PBMC donors). Using the available data from the other five donors, we found induction of IFN-γ production by the BF mixture in four of them (>100% v. control) and in three of them also with *B. breve* (>50% v. control), which is singularly high in the BF combination. Moreover, three donors showed stimulation with the FF mixture (>100% v. control). The BF mixture was the stimulus that induced the highest IFN-γ production (Fig. 2(C)), significantly higher than *B. adolescentis* (P=0.014), *B. infantis* (P=0.050) and *B. longum* (P=0.047) individually. Although *B. breve* also induced the production of IFN-γ, this effect was not significantly different from the other bifidobacteria (Fig. 2(C)). The effect of *B. adolescentis* on IFN-γ induction was inhibitory relative to the control condition and was significantly different from the stimulatory effect observed with the bifidobacteria mixtures and *B. longum* (Fig. 2(C)).

Cytokine production by Caco-2 cells in co-culture with peripheral blood mononuclear cells and bifidobacteria

To assess the effects of bifidobacteria and bifidobacteria mixture stimulation on Caco-2 cells in co-culture with PBMC, TNF-α, IL-1b, IL-10, IL-8 and IL-6 cytokines were measured in both apical and basolateral media. All cytokines were not detectable in the apical medium, while in the basolateral medium only IL-8 and IL-6 were in a measurable concentration range (IL-8, 120–14000

pg/ml; IL-6, 30–600 pg/ml). When Caco-2 cells were stimulated with the bifidobacteria alone, with no PBMC in the underlying compartment, the stimulation of both cytokines was three to four times lower than in the co-culture system (data not shown).

When in co-culture with PBMC, *B. breve* highly stimulated the production of IL-6 and IL-8 on Caco-2 cells (66.8 and 45.5 %, respectively; Fig. 3(A) and (B)). For IL-8, this production was significantly higher, compared with *B. adolescentis* (P=0.035), *B. infantis* (P=0.025) and the FF mixture (P=0.013) (Fig. 3(B)). Although the BF mixture also induced IL-6 and IL-8 production (36.0 and 20.7 %, respectively), these values were not significantly higher than those induced by the FF mixture (Fig. 3(A) and (B)). No significant differences were observed for IL-6 production between the different stimuli assayed (Fig. 3(A)). Considering the PBMC donors individually, IL-8 and IL-6 production stimulated by the FF mixture was positively and significantly correlated with IL-8 and IL-6 production stimulated by *B. infantis* ($P<0.001$ for both cytokines). On the other hand, IL-8 production stimulated by the BF mixture was correlated with B. angulatum, *B. breve* and B. catenulatum ($P<0.05$), and IL-6 stimulated by the BF mixture correlated with *B. adolescentis* and B. catenulatum ($P<0.05$).

DISCUSSION

Bifidobacterium strains have shown the capacity to modulate cytokine production by IEC, monocyte-derived dendritic cells and PBMC in in vitro experiments (1,8,9). Trying to define this immunomodulatory capacity seems relevant in order to understand their contribution to the establishment of mucosal tolerance and balanced intestinal immune responses in the early stages of life. Both these processes have been linked to the prevention of immune-mediated disorders later in life, such as allergies or inflammatory bowel disease (17,18). Several studies have evaluated the effect of different bifidobacteria in the production of cytokines by Caco-2 cells and PBMC (6,9,19-21), but according to our knowledge, this is the first time that the *Bifidobacterium* strains used in the present study have been employed in co-culture experiments, and that the mixtures in the proportions of a FF and BF infant's typical microbiota have been used to stimulate these cell types.

In the present study, the levels of several cytokines were measured in two different systems: (1) a direct stimulation of PBMC with bifidobacteria and (2) a PBMC/Caco-2 cell co-culture with bifidobacteria stimulating the top layer of Caco-2 cells, which, in turn, can interact with underlying PBMC through soluble mediators. Reciprocally, PBMC were able to influence Caco-2 cell activity as well. The profile of cytokine production by PBMC exposed directly to the *Bifidobacterium* strains shows relevant differences compared with the profile of cytokine production by PBMC in the co-

culture system, where Caco-2 cells constitute a physical barrier preventing the access of PBMC to the bifidobacteria. The first differential finding was that the level of cytokine production was much lower in the co-culture system. For instance, while three out of six cytokines measured were above 1000 pg/ml when both bifidobacterial mixtures were used, and two out of the remaining three gave results higher than 100 pg/ml in direct contact, only IL-6 by PBMC in the co-culture system gave results higher than 1000 pg/ml. It is worth noting that while in direct contact, IL-6 and TNF-α were the cytokines most highly induced, in the co-culture system, not only IL-6 but also IL-10 was the cytokine most highly produced by PBMC. In this sense, Niers et al. showed in a single culture system that the production of IL-10 by PBMC is boosted by several *Bifidobacterium* strains, and this down-regulates the production of TNF-α and IL-12p70 by these cells. When they used a monoclonal antibody against IL-10, they found a huge increase in the production of these inflammatory cytokines.

Different cytokines (IL-8 and IL-6) were also stimulated on Caco-2 cells, but only when they were previously co-cultured with PBMC; no cytokine production was measured if the Caco-2 cells were cultured alone with the *Bifidobacterium* strains. Therefore, the presence of PBMC is an essential factor for the sensitisation of Caco-2 cells to respond to bifidobacteria, which is presumably exerted by the communication between the two cell types through soluble mediators. In the present study and other studies, Caco-2 cells alone have been found to be hyporesponsive to bifidobacteria stimulation (22) and also to other probiotic bacteria (21,22). Moreover, since cytokine production by Caco-2 cells in the co-culture system was only detectable in the basolateral medium and not in the apical medium, it demonstrates a polarised secretion by Caco-2 cells, as have been found earlier by other authors (21). In a similar co-culture system, in which Caco-2 cells were stimulated with non-pathogenic *Escherichia co*li and *Lactobacillus sakei*, an induction of TNF-α secretion into the subepithelial compartment was observed, and this cytokine was signalled as the fundamental candidate for cellular crosstalk (21). In contrast, we found no detectable production of TNF-α, which might be explained by a differential effect from different bacterial species and strains.

Regarding the immunomodulatory effects of specific strains used in these experiments, the most relevant findings have been found regarding the immunostimulatory effects of *B. breve*. This strain stimulated most of the production of IL-8 and IL-6 on both Caco-2 cells and PBMC. In the microbiota of BF infants, *B. breve* is the most representative *Bifidobacterium* species (after *B. infantis*, common in all milk-fed babies), and this could explain the high IL-8 and IL-6 levels produced by Caco-2 and PBMC stimulated with the BF mixture. This link between *B. breve* and the BF mixture was supported by the correlation found between IL-8 levels produced by Caco-2 cells

stimulated by *B. breve* and the BF mixture. Moreover, *B. breve* and the BF mixture also stimulated the production of IL-10 and IFN-γ by PBMC (in co-culture with Caco-2 cells). All these observations might indicate that the proportion of different *Bifidobacterium* species is an important determinant of the overall contribution to the stimulation of cytokines on the intestinal mucosa. In this sense, it is interesting to note that there was a correlation between the relative inhibition of IL-8 production by Caco-2 cells induced by the FF mixture and *B. infantis*. It seems that the differences in the proportions of the different strains between the mixtures and the stimulatory/inhibitory capacities shown by the individual strains might explain the results found with their combinations in the BF and FF mixtures.

According to the results, *B. breve* induced a slight proinflammatory response, which could turn the mucosal immune system on stand-by and prevent the release of a severe inflammation. It has already been reported that infant from 4 to 6 months old, who daily consumed infant formula fermented with *B. breve* and *Streptococcus thermophilus*, presented less severe episodes of acute diarrhoea than the standard formula group (23). Furthermore, Li et al.(24) showed that the administration of *B. breve* to low-birth-weight infants was useful in promoting the colonisation by other bifidobacteria, which might contribute to the establishment of a healthier microbiota. More recently, it has been found that the administration of *B. breve* to pre-term infants can up-regulate transforming growth factor-b1 signalling and may possibly be beneficial in attenuating inflammatory and allergic reactions in these infants (25).

In allergic models, some probiotic bifidobacteria have the capacity to suppress IL-4 production, *in vitro* (16) and *in vivo* (26). We have observed that not all bifidobacterial species induce the same IL-4 production (Fig. 1(D)), indicating different effects of the interaction between bifidobacteria and PBMC related to the species.

Regarding the stimulation of the regulatory cytokine IL-10 by PBMC after direct stimulation with *B. longum*, a similar finding has been previously described by Medina et al., who found that several strains of *B. longum* are strong inducers of IL-10 secretion on PBMC. On the other hand, the finding that *B. infantis* is a weak inducer of cytokine secretion after direct stimulation of both PBMC and Caco-2 cells is in agreement with prior published results that have described that *B. infantis* attenuates baseline IL-8 secretion in HT-29 epithelial cells(5) as well as pro-inflammatory IL-17 production by murine splenocytes and dextran sodium sulphate-induced intestinal inflammation (27,28).

In conclusion, among the *Bifidobacterium* species tested, *B. breve* seems to be the most immunostimulatory strain in a co-culture system resembling the physiological layout of different cell types in the intestinal mucosa. The presence and relative proportions of different

Bifidobacterium species in the microbiota of BF and FF infants could be key factors defining the immunomodulatory effect of the gut microbiota in early life.

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Las tablas y figuras del presente artículo se presentan junto con el artículo original (en formato pdf) que se incluye en el ANEXO I.

DISCUSIÓN GENERAL

Este trabajo de investigación aborda el estudio de factores que influyen en el desarrollo del SI y la microbiota en edad temprana. Para ello se ha seleccionado una población de niños lactantes con riesgo de desarrollar una patología asociada al SI como es la EC. En la alteración de los mecanismos normales de funcionamiento del SI se sabe que participan diversos factores genéticos y ambientales, por lo que en el presente trabajo hemos intentado identificar posibles factores ambientales que pueden influir en la susceptibilidad a desarrollar EC, por sí mismos o mediante la interacción con factores genéticos (HLA-DQ) y/u otros factores ambientales.

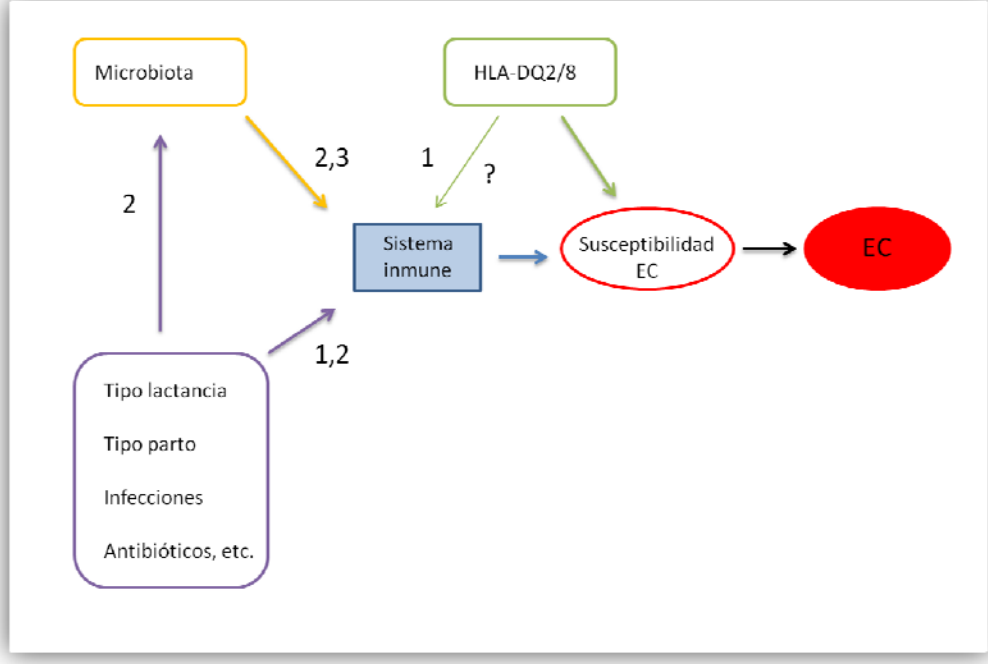

Figura 4. Diagrama de la posible relación entre el SI y desarrollo de EC con los factores ambientales y genéticos estudiados en este trabajo. 1) Estudio 1: *Influence of breastfeeding versus formula feeding on lymphocyte subsets in infants at risk of coeliac disease: the PROFICEL study*; 2) Estudio 2: *Influence of early environmental factors on lymphocyte subsets and gut microbiota in infants at risk of coeliac disease: the PROFICEL study*; 3) Estudio 3: *Immunostimulatory effect of faecal Bifidobacterium species of breast-fed and formula-fed infants in a peripheral blood mononuclear cell/Caco-2 co-culture system.*

1. EFECTO DE LOS FACTORES AMBIENTALES SOBRE EL DESARROLLO DEL SISTEMA INMUNE Y LA MICROBIOTA INTESTINAL

Dentro de los factores ambientales hemos seleccionado factores que pueden influir tanto en el desarrollo del SI, como en el establecimiento de la microbiota intestinal en edades tempranas, ya que ésta última está directamente relacionada con el SI, y su desequilibrio puede dar lugar a diversas enfermedades inmunológicas y puede aumentar la susceptibilidad a desarrollar EC. Estos factores ambientales seleccionados son, el tipo de lactancia, el tipo de parto, la toma de antibióticos por parte de la madre durante el embarazo y el parto, el desarrollo de infecciones y la toma de antibióticos durante los primeros 4 meses de vida, y la administración de la vacuna de rotavirus. Hemos evaluado el efecto de dichos factores ambientales sobre las subpoblaciones de linfocitos en sangre periférica y la microbiota de niños con 4 meses de edad y con riesgo genético de desarrollar EC.

Los resultados de los trabajos en los que se aborda este amplio objetivo indican que la mayor parte de los factores ambientales seleccionados ejercen un efecto sobre las subpoblaciones de linfocitos y la microbiota intestinal en nuestra población de estudio.

En un primer estudio vimos que los niños alimentados con leche materna mostraron un mayor porcentaje de células CD4+CD25+ y un menor número absoluto de células CD4+CD38+, comparado con los niños alimentados con leche de fórmula. Las células CD4+CD25+ son linfocitos T activados cuyo número aumenta con la exposición a antígenos, y CD38+ es un marcador constitutivo cuya expresión disminuye con la maduración. La naturaleza y función de estos marcadores sugiere que el SI se encontraría en un estado más avanzado de maduración en los niños con riesgo de EC que han sido alimentados con lactancia materna exclusiva durante los 4 primeros meses de vida. Esto indicaría una posible desventaja de introducir precozmente la lactancia de fórmula. La modulación ejercida por la leche materna sobre la subpoblación CD4+CD25+ parece relevante, y podría coincidir con otros estudios que han observado que la leche materna induce tolerancia oral y previene del desarrollo de asma y enfermedades autoinmunes a través del incremento de células T reguladoras CD4+CD25+Foxp3 [149-151]

En el segundo trabajo publicado se observó que la lactancia de fórmula se asoció con un mayor número de linfocitos totales, y número absoluto de células CD3+, CD4+, CD4+CD38+, CD4+CD28+, y CD3+CD4+CD45RO+. Existen otros estudios en niños de 6 meses de edad en los que han encontrado un mayor número de células T, en particular, colaboradoras (CD4+), en niños alimentados con leche de fórmula comparados con niños alimentados con lactancia materna. Por tanto, el tipo de lactancia es un factor importante a considerar dentro de los posibles factores

ambientales que pueden influir en el desarrollo del SI de niños con riesgo de EC y el subsiguiente posible desarrollo de la enfermedad. Además hemos investigado el efecto de las interacciones entre el genotipo HLA y el tipo de lactancia sobre las subpoblaciones linfocitarias de niños con riesgo de padecer EC a los 4 meses de edad. También se estudió el efecto individual del genotipo HLA. No encontramos interacción entre el tipo de lactancia y el genotipo HLA sobre las subpoblaciones de linfocitos. Por el contrario, sí encontramos efecto del genotipo HLA sobre el número absoluto de células NK. Los niños pertenecientes al grupo de riesgo intermedio mostraron un mayor número absoluto de células NK, comparado con los niños del grupo de alto y bajo riesgo. Este puede ser un resultado fortuito sin relevancia biológica, aunque existen estudios publicados sobre factores genéticos que influyen en la regulación de las subpoblaciones de linfocitos [152-155].

Otro resultado a destacar es que las infecciones sufridas por el niño durante los 4 primeros meses están asociadas a un aumento en el número de los linfocitos T, en particular los linfocitos CD4+, y dentro de éstos los CD3+CD4+CD45RO+, CD4+CD25+, y CD4+HLA-DR+. También, han mostrado estar asociadas a un número más alto de células NK, por lo que su efecto sobre el desarrollo del SI parece ser patente. Por el contrario, hemos visto que el desarrollo de alergia o dermatitis se asocia a un número más bajo de células NK. De acuerdo con esto y apoyado por el estudio de Han *y col.* [156], nuestros datos sugieren que las infecciones sufridas durante los primeros 4 meses de edad podrían proteger, a largo plazo, frente el desarrollo de alergias y dermatitis a través del efecto de las infecciones sobre las células NK.

En este trabajo, la vacuna frente al rotavirus, al contrario que la lactancia materna, se ha asociado a un menor porcentaje de células CD4+CD25+ y un mayor porcentaje de linfocitos B. EN concordancia con nuestros resultados, el estudio de Wang *y col.* [157] sugiere que la infección por rotavirus altera la homeostasis de las células T y el balance entre linfocitos T y B. Por tanto, la vacuna frente al rotavirus, al igual que la infección por rotavirus podría alterar el equilibrio entre células T y B en niños de 4 meses de edad con riesgo de desarrollar EC.

En relación con los resultados obtenidos del análisis de la microbiota intestinal de estos niños, cabe destacar que el parto por cesárea y la administración de antibióticos durante los primeros 4 meses de vida, han mostrado tener un efecto de detrimento del número de bifidobacterias. En especial, *B. catenulatum* en el caso del parto por cesárea, y *B. longum* en el caso de la toma de antibióticos en los primeros 4 meses de vida.

Una consideración importante es que nuestros resultados no se pueden extrapolar a la población general ya que el grupo de población seleccionado para el estudio está constituido por niños con riesgo familiar de desarrollar EC, en los que la distribución de genes como el

correspondiente al marcador HLA-DQ no es semejante a la de la población general. Por tanto, existe un sesgo de selección que impide la extrapolación de los resultados.

2. EFECTO INMUNOMODULADOR DE LA MICROBIOTA INTESTINAL *IN VITRO*

Hemos querido evaluar también el efecto inmunomodulador de la microbiota intestinal sobre el SI, para aportar más conocimientos al estudio de su contribución en el establecimiento de la tolerancia oral de la mucosa y el desarrollo de respuestas inmunológicas intestinales equilibradas en las primeras etapas de la vida. Para ello, hemos evaluado *in vitro* el efecto de diferentes cepas de bifidobacterias en la producción de citoquinas por parte de células Caco-2 y CMNs mediante dos sistemas de cultivo diferentes: (1) estimulación directa de CMNS con bifidobacterias y (2) co-cultivo de CMNs y células Caco-2 con bifidobacterias estimulando la parte apical de la monocapa de células Caco-2, las cuales, a su vez, pueden interactuar con las CMNs subyacentes a través de mediadores solubles y receptores de membrana en un sistema más aproximado a la situación fisiológica.

En nuestro trabajo hemos demostrado, *in vitro*, que las bifidobacterias características de la microbiota intestinal de niños con riesgo de EC alimentados con leche materna y leche de fórmula, tanto en combinación, como cada cepa por separado, ejercen diferentes efectos inmunoestimuladores sobre CMNs en un sistema de co-cultivo con células Caco-2, y que la presencia y las proporciones relativas de dichas bifidobacterias en la microbiota intestinal de estos niños puede ser un factor clave del efecto de la microbiota sobre el SI a edades tempranas.

El perfil de la producción de citoquinas por parte de las CMNs expuestas directamente a las cepas de bifidobacterias mostró diferencias relevantes comparadas con el perfil de la producción de citoquinas por parte de las CMNs en el sistema de co-cultivo con células Caco-2. En dicho sistema las células Caco-2 constituyen una barrera física para el acceso de las bifidobacterias a las CMNs. La primera diferencia es que los niveles de producción de citoquinas son más bajos en el sistema de co-cultivo. La segunda es que cuando usamos las dos mezclas de bifidobacterias, la mezcla de bifidobacterias característica de niños alimentados con leche materna (mezcla LM) y con leche de fórmula (mezcla LF), en contacto directo con las CMNs, las citoquinas más altamente inducidas fueron IL-6 y TNF-α, en cambio, en el sistema de co-cultivo fueron las citoquinas IL-6 e IL-10. Dentro de los resultados del cultivo directo con CMNs, se observó que la producción de IL-10 por parte de las CMNs, tal y como demostraron Niers *y col*. [84], es estimulada por ciertas cepas de *Bifidobacterium*, y esta producción inhibe la producción de TNF-α por las CMNs. Sin embargo, no se observaron diferencias en la producción de IL-10 estimulada por las diferentes cepas bacterianas o mezclas empleadas en el sistema de co-cultivo.

En el caso de la estimulación de la producción de citoquinas por parte de las células Caco-2, no se detectó producción de citoquinas cuando las células Caco-2 eran estimuladas sólo con bifidobacterias. Era necesaria la presencia de las CMNs para detectar producción de citoquinas (IL-8 e IL-6) por parte de las células Caco-2. Por lo que, la presencia de CMNs es un factor esencial para que las células Caco-2 pueden responder al estímulo de las bifidobacterias.

En relación con los efectos inmunomoduladores de cepas específicas, los resultados más relevantes se han encontrado con respecto a los efectos inmunoestimuladores de *Bifidobacterium breve*. Esta cepa estimuló, en general, la producción más alta de las citoquinas IL8- e IL-6 por parte de las células Caco-2 y las CMNs. En la microbiota de niños alimentados con leche materna, *B. breve* es la especie de bifidobacteria más representativa (después de *B. infantis*, que es la mayoritaria en todos los niños lactantes independientemente del tipo de lactancia recibida), por lo que esto puede explicar los altos niveles de IL-8 e IL-6 producidos por las células Caco-2 y las CMNs con la mezcla LM de bifidobacterias. Además, *B. breve* y la mezcla LM también estimularon la producción de IFN-γ por parte de las CMNs en co-cultivo con células Caco-2. Estas observaciones sugieren que la proporción de diferentes especies de bifidobacterias es un determinante importante para la contribución global de la estimulación de la producción de citoquinas sobre la mucosa intestinal. Parece que las diferencias entre las proporciones de diferentes bifidobacterias entre las mezclas LM y LF y las capacidades estimuladoras o inhibidoras mostradas por cada cepa individualmente, podrían explicar los resultados encontrados con cada una de las mezclas.

De acuerdo con nuestros resultados, *B. breve* induce una leve respuesta pro-inflamatoria que puede poner al SI de la mucosa en alerta y prevenir la formación de una fuerte respuesta inflamatoria en respuesta a antígenos externos. Existen varios estudios que han demostrado efectos beneficiosos de *B. breve* en niños lactantes [158-160].

En relación con la producción de la citoquina reguladora IL-10 por parte de las CMNs, cabe mencionar que después de estimulación directa con *B. longum* se producen altos niveles de dicha citoquina. Un resultado similar ha sido descrito previamente por Medina *y col.*, quienes encontraron que varias cepas de *B. longum* son fuertes inductores de la secreción de IL-10 en CMNs. Por otro lado, nuestra observación de que *B. infantis* es un débil inductor de la secreción de citoquinas después de la estimulación directa de CMNs y células Caco-2, concuerda con publicaciones previas en las que se describió que *B. infantis* atenúa la secreción basal de IL-8 en células epiteliales HT-29 [161], así como la producción de la citoquina pro-inflamatoria IL-17 por esplenocitos de ratón y la inflamación intestinal inducida por dextrán sulfato sódico [162, 163].

Por ello, esta bifidobacteria de presencia mayoritaria en lactantes podría tener un papel de contención de las respuestas inmunes en la mucosa intestinal.

3. POSIBLES MECANISMOS BIOLÓGICOS INVOLUCRADOS

La pregunta que surge ahora es qué mecanismos biológicos existen detrás de todo esto. La epidemia de enfermedades relacionadas con el SI en etapas tempranas de la vida es uno de los indicadores más claros de que el SI en desarrollo es vulnerable a los cambios ambientales modernos.

Estudios preliminares sugieren que los efectos de los factores ambientales tempranos sobre el sistema inmune podrían ser mediados epigenéticamente a través de una variedad de procesos que colectivamente modifican la expresión génica y la susceptibilidad a enfermedades relacionadas con el sistema inmune, y que estos efectos son potencialmente heredables a través de generaciones [164].

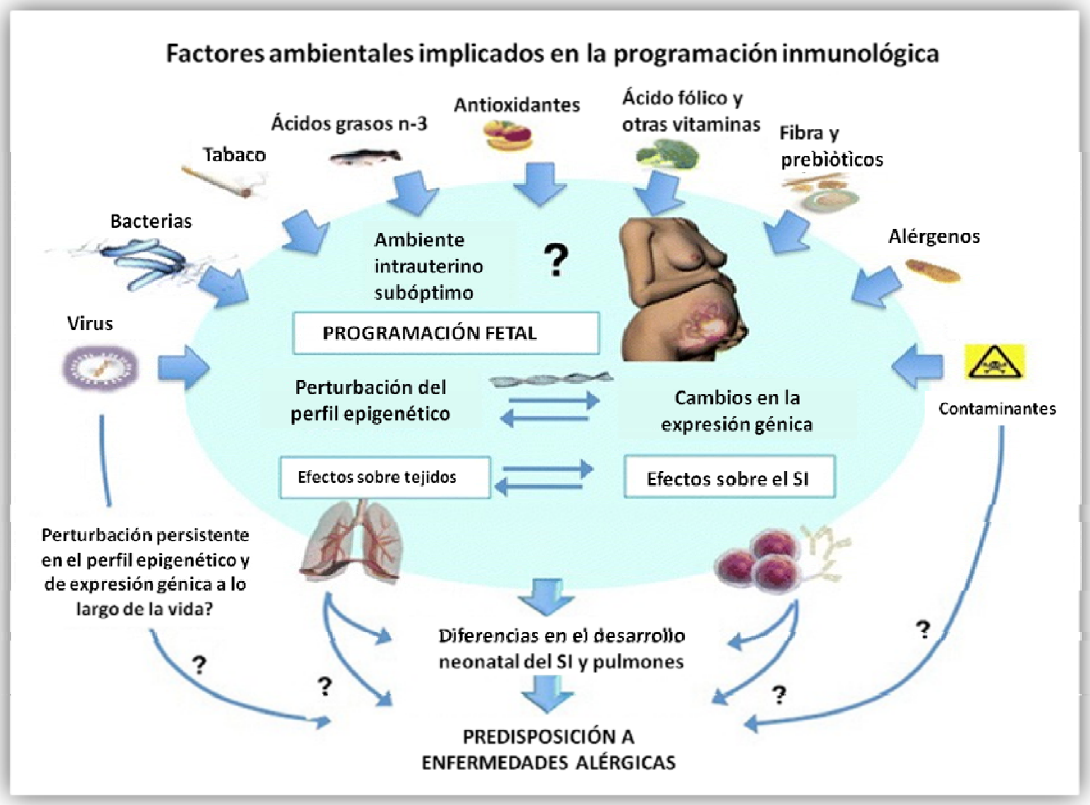

Figura 5. Perspectiva epigenética de la influencia de los factores ambientales tempranos sobre el desarrollo temprano del sistema inmune (figura tomada de *Prescott S y Saffery R, 2011*)

Lo esperado es que identificando los genes que están diferencialmente regulados en asociación con la subsecuente enfermedad ayudará en la identificación de las rutas y los factores ambientales que contribuyen al desarrollo de dicha enfermedad. Por eso, esto último, junto con la identificación de factores ambientales tempranos, es necesario para saber qué factores deben ser modificados para que se produzca un favorable desarrollo del SI, llevar a cabo nuevas estrategias de prevención, y así evitar la epidemia de enfermedades relacionadas con el SI, como es la Enfermedad Celíaca. En este trabajo se han podido identificar posibles factores ambientales tempranos que pueden afectar al desarrollo del SI y la microbiota, pero son necesarias más evidencias científicas para corroborar y definir con mayor claridad su papel en el desarrollo de futuras enfermedades inmunológicas.

A este respecto, el seguimiento de los niños de esta cohorte durante 3 o 5 años, y en definitiva, el mayor tiempo posible, permitirá realizar análisis discriminantes para identificar cuáles de los factores ambientales asociados con características distintivas de las subpoblaciones linfocitarias o la microbiota aumentan o disminuyen el riesgo de aparición de EC. Para ello es necesario conocer cuáles de los niños que han participado en este estudio desarrollarán la enfermedad.

4. ESTRATEGIAS DE PREVENCIÓN

Las estrategias de prevención de enfermedades asociadas al SI actuales son limitadas (evitar el tabaco, promoción de la lactancia materna, utilización de fórmulas hidrolizadas) [165], pero futuros estudios pueden proporcionar recomendaciones más claras acerca de la utilización de probióticos como estrategia de prevención.

En este trabajo hemos comprobado las características inmunomoduladoras interesantes que posee *Bifidobacterium breve* ATCC 15700 como posible cepa probiótica. También hemos comprobado que la presencia y las proporciones relativas de diferentes cepas de bifidobacterias (características de niños alimentados con leche materna o leche de fórmula) producen efectos diferentes en nuestro sistema de cultivo *in vitro*. Por todo ello, el siguiente paso que se deriva de nuestros estudios consistiría en seleccionar una cepa probiótica de *B. breve*, bien caracterizada, y realizar el primer estudio de intervención con probióticos en recién nacidos para comprobar la eficacia en la prevención de la aparición de la enfermedad en niños genéticamente predispuestos, que deberían recibir el probiótico desde el nacimiento o incluso realizando una suplementación durante la fase final del embarazo a la madre, tal y como se ha realizado en numerosos estudios de prevención en niños con riesgo de alergia.

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CONCLUSIONES

1. Los resultados obtenidos para las 20 diferentes subpoblaciones de linfocitos estudiadas concuerdan con los datos publicados en la literatura para niños sanos de 3 a 6 meses de edad, sin embargo, son los primeros datos publicados con esta completa batería de marcadores linfocitarios en niños españoles.

2. En los niños alimentados con lactancia materna exclusiva se ha observado un menor número de células CD4+CD38+ y un mayor porcentaje de células CD4+CD25+, lo que sugiere que la leche materna tiene un efecto potenciador sobre la maduración de los linfocitos. Este aumento de la subpoblación CD4+CD25+ parece ir en línea con la inducción de células T reguladoras que se atribuye a la lactancia.

3. No se han encontrado interacciones entre el genotipo HLA-DQ, indicativo del riesgo de padecer EC, y el tipo de lactancia en el efecto de estos factores sobre las subpoblaciones linfocitarias.

4. Las infecciones sufridas durante los primeros meses de vida se asociaron a un mayor número generalizado de células T colaboradoras, y en particular a los linfocitos T de memoria y los linfocitos T que expresan los marcadores de activación CD25+ y HLA-DR+, lo que refleja el papel de las infecciones en la maduración del SI.

5. Las infecciones sufridas durante los primeros meses de vida se asociaron a un mayor número de células NK. Por el contrario, el desarrollo de alergias o dermatitis en los niños dentro de los primeros 18 meses de vida se asoció a un menor número de células NK. Nuestros datos sugieren que las infecciones sufridas durante los primeros 4 meses de vida podrían proteger, a largo plazo, del desarrollo de alergia o dermatitis a través del efecto de las infecciones sobre las células NK.

6. El parto por cesárea y la toma de antibióticos durante los primeros 4 meses de vida se asociaron a un menor número de bifidobacterias. La influencia de estos dos factores en el establecimiento postnatal de la microbiota intestinal podría dar lugar a una regulación anómala del sistema inmune de la mucosa.

7. La influencia de las asociaciones encontradas entre los factores ambientales en edades tempranas, las subpoblaciones de linfocitos y el desarrollo de la microbiota sobre el riesgo de

padecer EC sólo se conocerán en el futuro, cuando estemos en condiciones de realizar análisis discriminantes una vez conocidos los casos de EC que hayan de aparecer en la cohorte de estudio.

8. El estudio *in vitro* de las características inmunomoduladoras de bifidobacterias demostró que la capacidad inmunomoduladora de las diversas cepas estudiadas difiere sensiblemente cuándo se estudia su efecto directo sobre CMNs y cuando se utiliza el sistema de co-cultivo, más semejante a la situación fisiológica de un intestino humano, dando a entender que la comunicación entre microbiota, células epiteliales intestinales y células inmunes residentes en la mucosa intestinal es muy relevante en la regulación del sistema inmune de la mucosa. Este tipo de sistema para la caracterización de la capacidad inmunomoduladora de potenciales cepas probióticas parece más adecuado que el cultivo directo de bacterias en contacto con CMNs.

9. La capacidad inmunomoduladora obtenida para las mezclas de bifidobacterias estudiadas, correspondientes a la microbiota típica de niños alimentados con lactancia materna y de fórmula, parecen ser consistentes con los efectos individuales de las cepas más características de cada una de ellas. Así, por ejemplo, *B. breve* y la mezcla LM estimularon la producción de IFN-γ por parte de las CMNs en co-cultivo con células Caco-2. De ello se desprende que la presencia y las proporciones relativas de las diferentes especies de *Bifidobacterium* en la microbiota de los niños alimentados con leche materna y leche de fórmula podrían ser factores clave que definen el efecto inmunomodulador de la microbiota intestinal en los primeros años de vida.

10. En nuestro estudio de co-cultivo *in vitro,* que se asemeja a la disposición fisiológica de los diferentes tipos de células en la mucosa intestinal, *Bifidobacterium breve* parece ser la cepa con mayor capacidad de inmunoestimulación, y sería de utilidad la realización de estudios *in vivo* con alguna cepa bien caracterizada de esta especie bacteriana en niños con riesgo de EC, preferiblemente en etapas tempranas de la vida, con el objetivo de comprobar los efectos beneficiosos frente a los mecanismos inmunopatogénicos de la enfermedad y la prevención de la misma.

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ANEXO I. Publicaciones originales

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ORIGINAL CONTRIBUTION

Influence of breastfeeding versus formula feeding on lymphocyte subsets in infants at risk of coeliac disease: the PROFICEL study

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Abstract

Purpose In addition to genetic risk, environmental factors might influence coeliac disease (CD) development. We sought to assess the effect of the interaction between milk-feeding practices and the HLA-DQ genotype on peripheral lymphocyte subsets and their activation markers in infants at familial risk for CD.

Methods 170 newborns were classified in 3 different genetic risk groups (high risk, HR; intermediate risk, IR; and low risk, LR) after DQB1 and DQA1 typing. Lymphocyte subsets were studied at the age of 4 months by flow cytometry analysis.

Results 79 infants were receiving exclusive breastfeeding (BF) and 91 partial breastfeeding or formula feeding (FF). Regarding genetic risk, 40 infants were classified in HR group, 75 in IR group and 55 in LR group. Two-way ANOVA did not show significant interactions between the type of milk feeding and genetic risk group on the lymphocyte subsets analysed. One-way ANOVA for milk-feeding practice alone showed that the percentage of CD4 + CD25+ cells was significantly higher in BF group than in FF group (BF, 10.92 ± 2.71; FF, 9.94 ± 2.96; $p = 0.026$), and absolute counts of CD4 + CD38+ cells were significantly higher in FF group than in BF group (FF, 2,881.23 ± 973.48; BF, 2,557.95 ± 977.06; $p = 0.038$).

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One-way ANOVA for genetic risk alone showed that absolute counts of NK cells were significantly higher in IR group than HR and LR groups (IR, 539.24 ± 340.63; HR, 405.01 ± 239.53; LR, 419.86 ± 262.85; $p = 0.028$).
Conclusion Lymphocyte subset profiles in the early stages of life could be modulated by milk-feeding practices and genetic risk separately. Breastfeeding might have a positive immunomodulatory effect on lymphocyte subsets in infants at risk of CD.

Keywords Lymphocyte subsets · Coeliac disease · Infants · HLA genotype · Breastfeeding · Formula feeding

Abbreviations

CD	Coeliac disease
PCR-SPP	Polymerase chain reaction-sequence-specific primers
HLA	Human leucocyte antigen
Treg	Regulatory T cells
NK	Natural killer
LR	Low genetic risk
IR	Intermediate genetic risk
HR	High genetic risk
BF	Breastfeeding
FF	Formula/mixture feeding

Introduction

Coeliac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of gluten in genetically susceptible individuals. It is generally accepted that CD is a T-cell-mediated disease, in which gliadin-derived peptides activate lamina propria infiltrating T lymphocytes. The subsequent release of pro-inflammatory cytokines, in particular γ-interferon, leads to a profound tissue remodelling. However, an innate immune response also seems to be involved in the disease development [1]. This is a complex disorder, with environmental and genetic factors contributing to its aetiology. The major genetic risk factor in CD is represented by HLA-DQ genes. Several studies have documented that the HLA-DQA1*05 and DQB1*02 alleles, encoding for particular DQ2 molecules, confer high susceptibility to CD. This heterodimer can be encoded both in cis and in trans. The susceptibility to CD is increased in homozygous subjects with a cis haplotype or possessing a second HLA-DQB1*02 allele [2–4]. In Europe, approximately 90 % of patients have these genetic markers, whereas most of the remaining cases carry the HLA-DQA1*03 and DQB1*0302 alleles coding for DQ8 molecules [3, 5]. Gluten is the main environmental factor responsible for the signs and symptoms of the disease. Moreover, the interplay between other environmental elements and the genetic background is also thought to play a role in the disease risk, including the type of milk feeding, incidence of infections and intestinal dysbiosis [6].

The effect of environmental factors on future disease risk is relevant at the early stages of life when the immature neonate's gut undergoes the process of microbiota establishment and the immune system acquires full competence and tolerance to nonharmful antigens [7]. A particular feature of the neonatal immune system is the presence of a wide pool of naïve T cells waiting to participate in primary immune responses. Intestinal antigen exposure during neonatal life influences appropriate adult immune responses. T-cell differentiation occurs within the neonatal human intestine, and the T-cell receptor (TcR) repertoire of these developing immature T cells is likely to be influenced by luminal antigens [8]. Oral ingestion of food and environmental bacteria is a major route of antigenic exposure. After birth, lactation supports immunological defences through a number of molecules with antimicrobial activity and immunologically active components present in breast milk [9, 10]. Thus, breastfeeding is known to confer protection against infectious diseases in the short term and there is also some evidence of lower prevalence of inflammatory bowel diseases, childhood cancers and type I diabetes in breastfed infants [11].

As early events during extra-uterine life allow the maturation of the immune system, the peripheral lymphocyte subsets also exhibit certain changes. Blood cell analysis shows marked lymphocytosis at birth and at later stages of life compared to adulthood. An increase in T and B lymphocytes occurs during the first weeks of life, while NK cells decline sharply directly after birth [12, 13]. A high CD4/CD8 ratio in babies up to 2 years of age has been found compared to the values observed in normal adults, and this is mainly due to the large and expanding pool of naïve T CD4+ cells present during the first year of life [14, 15]. The ectoenzyme CD38 is expressed in almost all T and B cells [12] and decreases towards the adult life [15, 16]. Although breastfeeding is likely to contribute to these changes, only a limited number of studies have addressed the influence of milk-feeding practices on peripheral lymphocyte subsets or the immunocompetence of lactating children [17–20] and these findings are not consistent among them.

Taking in consideration how important the maturation of the immune system can be as a predisposing or protective factor in future disease development, we planned to assess the effect of milk-feeding practices on the levels of lymphocyte subsets and their activation markers in infants at risk of CD. Since genetic background is a key issue for disease development in this population, we also aimed to

find out possible interactions between type of milk feeding and the HLA genotype on the lymphocyte subsets of these infants.

Materials and methods

Subjects

This study was conducted in 4-month-old infants who are first-degree relatives of CD patients (at least one parent or sibling affected with CD). A total of 170 infants born between October 2006 and the end of 2010 were recruited from 6 hospitals geographically distributed throughout Spain. The 170 infants were divided according to milk-feeding practices into two groups: breastfed (BF) infants ($N = 79$), including those which had been exclusively breastfed until the age of 4 months, and formula-fed (FF) infants ($N = 91$), including babies receiving milk formula, either alone or in alternation with breast milk at the moment of analysis. Questions about delivery, infections, vaccinations, feeding practices, clinical symptoms and medical treatments were answered by the parents at the infant's age of 1 and 4 months in clinical visits with the paediatric gastroenterologist. To facilitate the collection of this information, a diary was given to the mothers upon recruitment (before delivery). The study was approved by the local ethic committees, and written informed consent was obtained from the parents of children included in the study.

This cohort of infants was analysed for the HLA class II DQA1 and DQB1 genotypes. The infants were classified into three main risk groups, according to their DQ haplotype, loosely based on the criterion of Bourgey et al. and considering the HLA distribution of the Eastern Spanish population [21]. We established the following risk groups: The first one included those individuals carrying the DQ2 haplotype, both in *cis* (DQA1*0501-DQB1*0201 in homozygosis) and in *trans* (DQA1*0201-DQB1*0202 with DQA1*0505-DQB1*0301 in heterozygosis) conformation. The second group included those subjects carrying the DQ2 along with any other haplotype, as well as subjects carrying the DQ8 haplotype (DQA1*0301-DQB1*0302) in homozygosis. The third group included those individuals with other common genotypes not associated with CD. Probabilities are approximated and have been estimated taking into account that in most cases, the genotype of the coeliac relative of each newborn was not considered. 40 infants were in the first risk group (high risk; HR) with the highest probabilities of developing CD (28–24 %) [4]. In the second group, with a probability between 7 and 8 %, 75 of the infants were grouped (intermediate risk; IR), and the remaining 55 were in the third group (low risk; LR), with less than 1 % risk of developing CD.

DNA isolation

DNA was extracted from buccal mucosa cells by scraping the inner side of the children's cheek with sterile swabs (Copan innovation, Sarstedt, Germany). The cotton was cut and incubated in DLB buffer (100 mM Tris–HCl pH 7.4, 10 mM EDTA pH 8, 10 mM NaCl per litre of distilled water), 10 % SDS and 10 µL proteinase K (20 mg/ml) at 65 °C for 1 h, and then, a standard phenol–chloroform method was carried out. Extracted DNA was stored in TE buffer (10 ml Tris–HCl pH 8, 200 µL EDTA 0.5 M pH 8 per litre of distilled water) at −20 °C after genotyping. The DNA concentration, around 50–100 µg/mL, was quantified using the NanoDrop© Spectrophotometer.

HLA-DQ genotyping

Low-resolution HLA-DQB1 typing was performed by PCR-SSP (polymerase chain reaction-sequence-specific primers) analysis. Each PCR was performed on about 60–90 ng of extracted DNA, 0.5 U of BIOTOOLS DNA polymerase (Biotools B&M S.A, Spain), 1 × PCR Master Mix (Dynal AllSet + TM SSP or Olerup SSPTM) containing nucleotides (200 µmol L^{-1} each), PCR buffer (50 mmol L^{-1} KCl, 1.5 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} Tris–HCl pH 8.3, 0.001 % w/v gelatine), 5 % glycerol and 100 µg mL^{-1} cresol red, 0.25 µmol L^{-1} of each allele- or group-specific primer pair and 0.1 µmol L^{-1} of internal positive control primer pair matching a segment of the human growth hormone gene in a final volume of 10 µL. Detailed PCR protocol: An initial denaturation step at 94 °C for 2 min was followed by 10 two-temperature cycles (94 °C for 10 s and 65 °C for 60 s) and 20 three-temperature cycles (94 °C for 10 s, 61 °C for 50 s and 72 °C for 30 s). Detection of amplified alleles was carried out by 2 % agarose gel electrophoresis and ethidium bromide staining. Although the allele DQB1*02 is a determinant of DQ2, HLA-DQA1 alleles were genotyped in a stepwise fashion for a high-resolution typing to sharpen the risk classification of each individual.

Blood analyses

At the age of 4 months, peripheral blood samples were drawn from the infants and collected in vacutainer tubes containing K_3EDTA. Immediately after, 1 mL of blood was mixed with an equal volume (1 mL) of preservative solution (Streck Cell Preservative™ CE. Streck, USA) and sent in cool temperature within 2–6 days to our laboratory for a centralised processing and flow cytometry analysis. Blood extractions were always performed previous to the standardised 4-month vaccination in the Spanish vaccination schedule. Complete blood counts and differential

counts were performed in situ at the corresponding enrolling centres by automated instrumentation. Blood samples were not taken if the infant presented an ongoing infection or had received a vaccination shot in the previous 6 weeks.

Flow cytometry analysis

Aliquots of blood mixed with the preservative solution (150 µL) were incubated for 30 min at room temperature and in the dark with 20 µL of fluorochrome-conjugated monoclonal antibodies specific for CD3+ (CD3-APC), CD4+ (CD4-PerCP-Cy5.5), CD8+ (CD8-PerCP-Cy5.5), CD45RA+ (CD45RA-FITC), CD45RO+ (CD45RO-PE), CD25+ (CD25-FITC), HLA-DR+ (HLA-DR-FITC), CD38+ (CD38-PE), in quadruple immunostainings, and 100 µL aliquots were incubated with the multitests CD3-FITC/CD16 + 56-PE/CD45-PerCP-Cy5.5/CD19-APC and CD3-FITC/CD8-PE/CD45-PerCP-Cy5.5/CD4-APC. Fluorochrome-conjugated isotype control immunoglobulins (IgG1 and IgG2a) from mouse were used for each monoclonal antibody to avoid any background fluorescence signal due to nonspecific binding. All the monoclonal antibodies were purchased from Becton–Dickinson (Sunnyvale, CA, USA). After incubation, samples were lysed with the BD FACS™ Lysing Solution (Becton–Dickinson) following the manufacturer's protocol. The samples were analysed with FACSCalibur Flow Cytometer (four-colour, dual-laser, Becton–Dickinson). The lympho gate was defined on the forward and side scatter patterns of lymphocytes. The analysis protocol gated on lymphocytes stained with PerCP and/or APC, and the selected population was then analysed with the two remaining colours (FITC and PE) to obtain cell percentages expressing the specific antigens. Cell subset counts were obtained by multiplying subset percentages times anchor marker counts, the later resulting by multiplying subset percentage times the absolute lymphocyte count.

Statistics

The normality of the distribution of variables was confirmed through normality graphs and Shapiro–Wilk tests. All variables fitted normal distribution. A univariant analysis of variance was employed with HLA risk and milk-feeding groups as fixed factors to assess the interaction between HLA status and type of milk feeding. When a significant interaction was found, differences between milk-feeding groups were analysed by t test comparison of means within each risk level, and differences between risk groups by one-way ANOVA within each feeding type level followed by post hoc analysis. When no interaction was obtained between the two factors, the analyses were carried out for each factor separately. A p value lower than 0.050 was considered statistically significant (Fig. 1).

Results

The demographic characteristics of the infants included in the study are presented in Table 1. All infants were full-term (39.02 ± 1.77 weeks of gestation), and the majority of them were vaginally delivered (118 out of 170). The size and the weight of the infants at the moment of the delivery were within standard ranges and did not differ significantly between the groups.

The percentage and cell counts of the main lymphocyte subsets and subsets expressing activation markers in the whole group of 4-month-old infants are shown in Table 2. Two-way ANOVA for type of milk feeding and genetic risk group did not show significant interactions between genetic risk and milk-feeding practices on the lymphocyte subsets analysed.

Two effects of the milk-feeding type alone were found (Table 3). Firstly, one-way ANOVA for milk-feeding practice alone showed that the percentage of CD4 + CD25+ cells was significantly higher in BF group than in FF group (BF, 10.92 ± 2.71; FF, 9.94 ± 2.96; $p = 0.026$), and secondly, absolute counts of CD4 + CD38 + cells were significantly higher in FF group than in BF group (FF, 2,881.23 ± 973.48; BF, 2,557.95 ± 977.06; $p = 0.038$). The effects of HLA genotype alone were also analysed (Table 4). One-way ANOVA for genetic risk alone showed that absolute counts of NK cells were significantly higher in IR group than HR and LR groups (IR, 539.24 ± 340.63; HR, 405.01 ± 239.53; LR, 419.86 ± 262.85; $p = 0.028$).

Discussion

In this study, the combined effect of HLA-DQ genotype and milk-feeding practices at 4 month of age on peripheral lymphocyte subsets was studied in a group of infants with at least one first-degree relative suffering from CD. The infants' HLA genotype was classified into genetic risk groups in concordance with the last results obtained from the Italian and Spanish population distribution [4, 21]. However, taking into account our sample size, the five-group initial classification was reduced to a three-group one. Our analysis did not reveal any interaction between type of feeding and genotype on lymphocyte subsets' profile, but both factors showed an independent effect on specific T-cell subsets.

The results obtained for the 20 different lymphocyte subsets measured (median and 10th and 90th percentiles) are in agreement with data published in the literature for

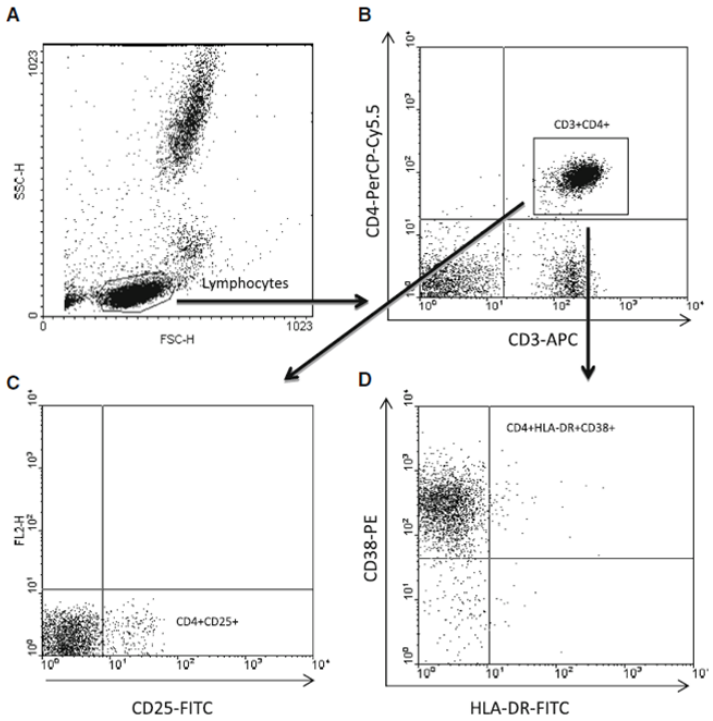

Fig. 1 Four-colour flow cytometry analysis of total blood from a 4-month-old infant. a Lymphocytes were gated in the forward scatter (FSC) and side scatter (SSC) histogram according to the size and complexity of the total white blood cells. b Helper T cells (CD3 + CD4+) were gated and used as the anchor marker to analyse CD4 + CD25+ c and CD4 + CD38+ and CD4 + HLA-DR + CD38+ (d)

Table 1 Demographic variables in coeliac disease risk infants

	Total subjects (n = 170)
Delivery	
Vaginal	118/170
Caesarean	52/170
Size (cm)	49.73 ± 2.32
Weight (g)	3,270.57 ± 458.64
Weeks of gestation	39.02 ± 1.77
Infants at 4 months of age	
Breastfeeding	Subjects (n)
Exclusive at 4 months	79
Formula feeding	Subjects (n)
Formula before 4 months	91

3- to 6-month-old healthy infants [15, 22]. However, no reference data have been published so far for this age range in the Spanish population.

A role for breastfeeding as an environmental factor with the capacity to produce immune modulation that might perhaps delay or reduce the risk of developing CD has been suggested by several authors [23–25]. In our population of infants at risk, we have only found a slight modulation of the lymphocyte subset counts by breastfeeding, since most of the subsets did not show differences between breastfed and formula-fed infants. In the literature, the effects of breastfeeding *versus* formula feeding on lymphocyte subsets are controversial, but two different studies have found an increased percentage of CD8+ cells in breastfed infants at 6 and 8 months, compared to formula-fed infants [19, 20]. In our infants, no differences were found in the CD4+ and CD8+ lymphocytes; however, we found lower CD4 + CD38+ counts and higher CD4 + CD25+ percentage in breastfed infants compared to FF infants. CD38+ has a role in T-cell activation and differentiation [26] and is constitutively expressed in newborns decreasing towards the adult life. The fact that lower CD4 + CD38+ counts are found in breastfed children supports the enhancing effect of breastfeeding on lymphocyte maturation.

Regarding the lower CD4 + CD25+ percentage with formula feeding, this might reflect a lower number of regulatory T cells in these infants. However, since we did not use a specific Treg marker, a major drawback of our

Table 2 Cell subset percentages and counts of peripheral blood lymphocytes in 4-month-old infants at risk of coeliac disease

Subsets	Relative frequencies (%) $N = 170$	Absolute counts (cells/μL) $N = 163$
CD45 + CD3+	65 (55–74)	3,706 (2,563–5,780)
Mature T cells		
CD45 + CD4+	46 (38–56)	2,719 (1,816–4,255)
Helper T cells		
CD45 + CD8+	14 (10–22)	831 (520–1,439)
Cytotoxic T cells		
CD45 + CD3-(CD56 + 16)+	6 (4–12)	373 (189–949)
NK cells		
CD45 + CD19+	23 (15–34)	1,291 (755–2,206)
Mature B cells		
CD4 + CD45RA+	87 (77–92)	2,264 (1,453–3,684)
Naïve T4 cells		
CD8 + CD45RA+	90 (77–96)	722 (447–1,217)
Naïve T8 cells		
CD3 + CD45RO+	12 (7–23)	446 (264–918)
Pan Memory T cells		
CD3 + CD4 + CD45RO+	9 (5–14)	321 (190–516)
Memory T4 cells		
CD3 + CD8 + CD45RO+	2 (1–7)	81 (36–277)
Memory T8 cells		
CD4 + CD25+	10 (7–14)	271 (184–439)
Activated T4 cells		
CD8 + CD25+	1 (0–4)	11 (4–35)
Activated T8 cells		
CD4 + HLA-DR	3 (2–6)	79 (42–179)
Activated T4 cells		
CD4 + CD38+	96 (93–98)	2,538 (1,645–4,112)
Activated T4 cells		
CD4 + HLA-DR + CD38+	2 (1–5)	65 (31–165)
Activated T4 cells		
CD8 + HLA-DR+	4 (2–16)	40 (14–183)
Activated T8 cells		
CD8 + CD38+	95 (89–98)	786 (481–1,404)
Activated T8 cells		
CD8 + HLA-DR + CD38+	4 (2–16)	37 (11–166)
Activated T8 cells		
CD4 + CD28+	100 (99–100)	2,719 (1,787–4,245)
Activation primed T4 cells		
CD8 + CD28+	95 (72–98)	747 (469–1,219)
Activation primed T8 cells		
Ratio CD4 + /CD8+	3 (2–5)	
Lymphocytes		5,805 (3,930–8,500)

Values are medians (10th and 90th percentiles) of the relative frequencies or absolute counts of cells expressing the indicated markers referred to the lymphocyte population defined by the anchor marker, which appears in first place of the subset name

study is the impossibility to differentiate within the CD4 + CD25+ population what proportion is activated to produce Th1 cytokines and what proportion is Treg to produce IL-10 and TGF-β. The importance of the Treg cells in the susceptibility of atopic disease in children with a familial history of the disease has been pointed out recently [27], even though their role is still not well defined. The modulation exerted by breastfeeding on this population seems relevant, since different animal and human studies have revealed that breast milk induces oral

Table 3 Lymphocyte subset percentages and counts in coeliac disease risk infants classified into groups according to milk-feeding practice

Lymphocyte subsets		Breastfeeding ($N = 79$)	Formula feeding ($N = 91$)	p
CD45 + CD3+	%	64 ± 8	65 ± 7	0.626
Mature T cells	cel./μL	3,824 ± 1,386	4,055 ± 1,224	0.260
CD45 + CD4+	%	46 ± 7	47 ± 7	0.264
Helper T cells	cel./μL	2,726 ± 945	2,991 ± 989	0.086
CD45 + CD8 +	%	16 ± 5	15 ± 5	0.262
Cytotoxic T cells	cel./μL	956 ± 513	925 ± 388	0.670
CD45 + CD3-(CD56 + 16)+	%	8 ± 4	7 ± 3	0.085
NK cells	cel./μL	494 ± 346	447 ± 253	0.317
CD45 + CD19+	%	23 ± 8	24 ± 7	0.767
Mature B cells	cel./μL	1,386 ± 649	1,489 ± 666	0.320
CD3 + CD45RO+	%	14 ± 7	14 ± 8	0.747
Pan memory T cells	cel./μL	518 ± 359	583 ± 431	0.307
CD4 + CD45RA+	%	86 ± 6	85 ± 9	0.594
Naïve T4 cells	cel./μL	2,352 ± 887	2,568 ± 908	0.136
CD3 + CD4 + CD45RO+	%	9 ± 4	10 ± 5	0.423
Memory T4 cells	cel./μL	331 ± 202	392 ± 263	0.109
CD8 + CD45RA+	%	88 ± 9	87 ± 9	0.711
Naïve T8 cells	cel./μL	832 ± 456	789 ± 277	0.468
CD3 + CD8 + CD45RO+	%	3 ± 3	3 ± 4	0.493
Memory T8 cells	cel./μL	125 ± 151	149 ± 209	0.417
CD4 + CD25+	%	11 ± 3	10 ± 3	**0.026**
Activated T4 cells	cel./μL	287 ± 91	289 ± 106	0.889
CD8 + CD25+	%	2 ± 1	2 ± 2	0.321
Activated T8 cells	cel./μL	16 ± 16	18 ± 21	0.461
CD4 + HLA-DR	%	4 ± 2	4 ± 2	0.919
Activated T4 cells	cel./μL	93 ± 54	103 ± 61	0.315
CD4 + CD38+	%	94 ± 11	96 ± 2	0.065
Activated T4 cells	cel./μL	2,558 ± 977	2,881 ± 973	**0.038**
CD4 + HLA-DR + CD38+	%	3 ± 2	3 ± 2	0.619
Activated T4 cells	cel./μL	76 ± 52	88 ± 56	0.173
CD8 + HLA-DR+	%	8 ± 10	7 ± 7	0.637
Activated T8 cells	cel./μL	93 ± 181	72 ± 110	0.375
CD8 + CD38+	%	93 ± 11	94 ± 5	0.196
Activated T8 cells	cel./μL	893 ± 503	869 ± 341	0.723
CD8 + HLA-DR + CD38+	%	7 ± 10	7 ± 6	0.663
Activated T8 cells	cel./μL	88 ± 175	67 ± 99	0.346
CD4 + CD28+	%	100 ± 1	100 ± 1	0.900
Activation primed T4 cells	cel./μL	2,718 ± 944	2,985 ± 992	0.083
CD8 + CD28+	%	90 ± 13	90 ± 13	0.794
Activation primed T8 cells	cel./μL	840 ± 437	815 ± 293	0.672
Ratio CD4 + /CD8+	%	3 ± 1	3 ± 1	0.295
Lymphocytes	cel./μL	5,985 ± 2,036	6,308 ± 1,876	0.294

Data are mean ± SD. One-way ANOVA ($p < 0.05$)

Significant differences are highlighted in bold

tolerance and prevents asthma and other autoimmune disorders development through an increase in CD4 + CD25 + Foxp3 + regulatory T cells [28–30]. Several in vitro studies showed that specific milk components, like exosomes [31], *lactobacilli* [32] and lactadherine milk protein [33], activate or increase the number of regulatory T cells (CD4 + CD25 + Foxp3+). Lactadherine, in addition, has been involved in protection against rotavirus

Table 4 Lymphocyte subset percentages and counts in coeliac disease risk infants classified into groups according to genetic risk

Lymphocyte subsets		High risk ($N = 40$)	Intermediate risk ($N = 75$)	Low risk ($N = 55$)	p
CD45 + CD3+	%	66 ± 7	64 ± 8	64 ± 8	0.406
Mature T cells	cel./μL	4,008 ± 1,419	4,149 ± 1,391	3,621 ± 1,017	0.079
CD45 + CD4+	%	48 ± 8	46 ± 7	47 ± 7	0.491
Helper T cells	cel./μL	2,880 ± 1,058	3,002 ± 1,038	2,678 ± 799	0.188
CD45 + CD8+	%	15 ± 5	15 ± 5	15 ± 5	0.872
Cytotoxic T cells	cel./μL	942 ± 470	1,014 ± 511	834 ± 302	0.086
CD45 + CD3-(CD56+16)+	%	6 ± 2	8 ± 4	7 ± 4	0.090
NK cells	cel./μL	405 ± 240	539 ± 341	420 ± 263	**0.028**
CD45 + CD19+	%	23 ± 7	23 ± 8	24 ± 7	0.666
Mature B cells	cel./μL	1,365 ± 687	1,490 ± 633	1,429 ± 678	0.633
CD3 + CD45RO+	%	15 ± 9	14 ± 8	13 ± 5	0.462
Pan memory T cells	cel./μL	603 ± 461	587 ± 430	468 ± 284	0.183
CD4 + CD45RA+	%	85 ± 10	85 ± 7	87 ± 5	0.477
Naïve T4 cells	cel./μL	2,475 ± 1,032	2,572 ± 960	2,325 ± 697	0.333
CD3 + CD4 + CD45RO+	%	10 ± 7	9 ± 4	9 ± 4	0.510
Memory T4 cells	cel./μL	395 ± 327	379 ± 202	320 ± 203	0.263
CD8 + CD45RA+	%	86 ± 10	87 ± 10	88 ± 7	0.427
Naïve T8 cells	cel./μL	806 ± 393	860 ± 412	738 ± 272	0.200
CD3 + CD8 + CD45RO+	%	4 ± 3	3 ± 4	3 ± 2	0.418
Memory T8 cells	cel./μL	160 ± 163	155 ± 239	98 ± 79	0.174
CD4 + CD25+	%	10 ± 3	11 ± 3	10 ± 3	0.167
Activated T4 cells	cel./μL	269 ± 94	307 ± 92	275 ± 110	0.083
CD8 + CD25+	%	2 ± 2	2 ± 1	2 ± 2	0.362
Activated T8 cells	cel./μL	15 ± 23	20 ± 20	13 ± 11	0.084
CD4 + HLA-DR	%	3 ± 2	4 ± 2	4 ± 2	0.532
Activated T4 cells	cel./μL	90 ± 58	104 ± 62	96 ± 53	0.486
CD4 + CD38+	%	96 ± 2	94 ± 11	96 ± 2	0.332
Activated T4 cells	cel./μL	2,777 ± 1,049	2,829 ± 1,080	2,567 ± 777	0.328
CD4 + HLA-DR + CD38+	%	3 ± 2	3 ± 2	3 ± 1	0.642
Activated T4 cells	cel./μL	77 ± 56	88 ± 59	79 ± 46	0.479
CD8 + HLA-DR+	%	7 ± 9	7 ± 9	7 ± 7	0.973
Activated T8 cells	cel./μL	93 ± 195	90 ± 156	62 ± 76	0.488
CD8 + CD38+	%	94 ± 5	93 ± 12	95 ± 3	0.376
Activated T8 cells	cel./μL	888 ± 454	941 ± 477	791 ± 289	0.146
CD8 + HLA-DR + CD38+	%	7 ± 9	7 ± 9	7 ± 7	0.966
Activated T8 cells	cel./μL	89 ± 1,888	84 ± 146	57 ± 72	0.481
CD4 + CD28+	%	100 ± 1	100 ± 1	100 ± 1	0.969
Activation primed T4 cells	cel./μL	2,873 ± 1,058	2,996 ± 1,038	2,671 ± 799	0.187
CD8 + CD28+	%	89 ± 13	89 ± 15	92 ± 9	0.349
Activation primed T8 cells	cel./μL	807 ± 310	883 ± 434	762 ± 283	0.177
Ratio CD4 + /CD8+	%	3 ± 1	3 ± 1	3 ± 1	0.709
Lymphocytes	cel./μL	6,107 ± 2,075	6,489 ± 2,020	5,745 ± 1,702	0.110

Data are mean ± SD. One-way ANOVA ($p < 0.05$)

Significant differences are highlighted in bold

infection [34–37]; some investigations have linked this pathogen to coeliac disease development [38].

Overall, our results showing a percentage of T CD4 + CD25+ cells significantly higher in BF group than in FF group, and absolute counts of T CD4 + CD38+ cells significantly higher in FF group than in BF group, suggest that breastfed infants could have a more mature immune system that formula-fed infants due to the beneficial

properties of human milk. This would be an argument in favour of the recommendation to introduce gluten in predisposed infants while they are still breastfed as a means to possibly lessen the toxicity of gliadin peptides [39]. However, since at the time of our lymphocyte subset analysis, gluten introduction had not occurred yet, it will be interesting to find out whether this apparently increased number of regulatory T-cell clones is associated with a better response to gliadin after gluten introduction. In addition, there are studies that demonstrate that breast milk contains gliadin peptides [40] and gliadin-specific IgA antibodies [40–42] that could be involved in the modulation of the immune response in neonates [40], possibly with a preventive role [42].

Regarding higher absolute counts of NK cells in IR group than HR and LR groups, the possibility exists that this is only a spurious finding, with no biological relevance. However, the association of specific genotypes with a certain marker, a function or a disease, is usually difficult to explain. In this sense, the concept that genetic factors influence the regulation of lymphocyte subpopulations has been supported by several published studies [43–46]. Therefore, more research is needed to clarify the true meaning of the association between NK cells and HLA-DQ genotype in the population studied.

In conclusion, according to our results, the effect of breast milk on lymphocyte subsets could be beneficial in infants at risk of CD and further studies are necessary to assess the combined effect of milk-feeding practices and gluten introduction practices on that risk.

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Conflict of interest The authors declare that they have no conflict of interest.

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Immunostimulatory effect of faecal *Bifidobacterium* species of breast-fed and formula-fed infants in a peripheral blood mononuclear cell/Caco-2 co-culture system

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Abstract

Bifidobacterium spp. typical of the human intestinal microbiota are believed to influence the balance of immune responses in the intestinal mucosa. The aim of the present study was to investigate the effect of different bifidobacterial species and their mixtures in *in vitro* experiments with peripheral blood mononuclear cells (PBMC) and Caco-2 cells. *Bifidobacterium adolescentis*, *B. angulatum*, *B. breve*, *B. catenulatum*, *B. infantis*, *B. longum* and two combinations of these bifidobacteria simulating the species composition found in faecal samples from breast-fed (BF) and formula-fed (FF) infants were used. The levels of several cytokines were measured by direct stimulation of PBMC and by stimulation of a Caco-2/PBMC co-culture with bifidobacteria. *B. catenulatum* and *B. breve* were the strongest enhancers of interferon-γ (IFN-γ) production by direct stimulation of PBMC. *B. longum* was the highest inducer of IL-10 and the lowest TNF-α stimulus. In the Caco-2/PBMC system, *B. breve* was the highest inducer of IL-8 production by Caco-2 cells, significantly different from *B. infantis*, *B. adolescentis* and the FF mixture ($P<0·05$). IFN-γ produced by PBMC stimulated with the BF mixture (containing 22 % *B. breve*, compared with 7 % in the FF mixture) was significantly higher compared with *B. adolescentis*, *B. infantis* and *B. longum*. *B. adolescentis* also inhibited IFN-γ production compared with the FF mixture and *B. longum*. The proportion of different *Bifidobacterium* strains seems to be an important determinant of the cytokine balance in the simulated intestinal environment studied. *B. breve* and the combination of the *Bifidobacterium* species typically found in the microbiota of BF infants have shown the most significant effects.

Key words: *Bifidobacterium* spp.: Caco-2 cells: Peripheral blood mononuclear cells: Cytokines: Breast-feeding: Formula feeding: Infant's microbiota

The intestinal microbiota plays a pivotal role in human health by preventing pathogen colonisation, and shaping and maintaining normal mucosal immunity[1]. To preserve this beneficial relationship, the immune system should remain hyporesponsive to commensal bacteria (mucosal tolerance)[2,3], but at the same time, it has to combat pathogenic bacteria[3]. The breakdown of the delicate balance of the intestinal immune responses causes the development of disease states with bowel inflammation[3]. In this context, intestinal epithelial cells (IEC) play an important role in immune homeostasis[4,5]. IEC are thought to contribute to immunomodulation of mucosal leucocytes by at least two different mechanisms[6], by acting as a physical barrier between gut luminal content (including bacteria) and the underlying immune cells, and by transmitting signals coming from the intestinal content and microbiota to the resident mucosal immune system[4]. IEC secrete many mediators involved in protective responses against potentially pathogenic organisms, such as defensins, mucins, chemokines and cytokines[5].

Bifidobacteria, which are important components of the human intestinal microbiota particularly of breast-fed (BF) infants[7], have shown the capacity to modulate cytokine production by IEC, monocyte-derived dendritic cells and peripheral blood mononuclear cells (PBMC) in *in vitro* experiments[1,8,9]. In addition, the differences observed in the composition of bifidobacterial species of the intestinal microbiota of BF and formula-fed (FF) infants have been suggested to influence the incidence of immune-mediated diseases[10,11]. These findings have led to the proposal to

Abbreviations: BF, breast-fed; FF, formula-fed; IEC, intestinal epithelial cells; IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cells.

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use some *Bifidobacterium* strains as potential probiotics in the prevention and treatment of pathologies with underlying immune alterations, such as inflammatory bowel diseases, allergy and coeliac disease[12–14].

Following all of the aforementioned facts and hypothesis, the objective of the present study was to investigate the effect of strains of different bifidobacterial species (*Bifidobacterium adolescentis*, *B. angulatum*, *B. breve*, *B. catenulatum*, *B. infantis* and *B. longum*) and their mixtures, corresponding to the typical microbiota present in the faeces from BF and FF children, on the modulation of cytokine production by IEC and PBMC in an *in vitro* co-culture system, simulating the intestinal environment.

Materials and methods

Bacteria

The following strains of six different *Bifidobacterium* species were individually evaluated: *B. adolescentis* ATCC 15703; *B. angulatum* ATCC 27535; *B. breve* ATCC 15700; *B. catenulatum* LMG 11043; *B. longum biovar infantis* LMG 11046T; *B. longum biovar longum* ATCC 15707. In addition, two combinations of these bifidobacteria were also used to simulate the percentage of each species in the microbiota from BF and FF infants[10]. The BF mixture included *B. infantis* (59·0%), *B. breve* (21·6%), *B. longum* (13·5%), *B. catenulatum* (3·5%), *B. angulatum* (1·8%) and *B. adolescentis* (0·6%); the FF mixture included *B. infantis* (62·1%), *B. catenulatum* (14·8%), *B. longum* (10·9%), *B. breve* (7·2%) and *B. adolescentis* (5·0%) (no *B. angulatum*).

Bifidobacteria were grown routinely in de Man Rogosa and Sharpe agar (Scharlau Chemie SA, Barcelona, Spain) with 0·05% cysteine broth and incubated at 37°C under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK) for 22 h. Cells were harvested by centrifugation (6000 ***g*** for 15 min) until the stationary growth phase, washed two times in PBS (130 mM-NaCl, 10 mM-sodium phosphate, pH 7·4, and resuspended in PBS containing 20% glycerol). Aliquots of these suspensions were frozen in liquid N_2 and stored at −80°C until used. The number of live cells after storage was determined by colony-forming unit counting on de Man Rogosa and Sharpe-cysteine after 48 h incubation in optimal conditions. For all strains tested, >90% of cells were alive upon thawing. For every new experiment, one fresh aliquot was thawed to avoid variability in cultures between the experiments.

Leucocyte isolation and bacterial stimulation of peripheral blood mononuclear cells

Human PBMC from seven healthy volunteers were isolated from heparinised blood samples using standard Ficoll gradient centrifugation (lymphocyte isolation solution; Rafer, Zaragoza, Spain). The isolated PBMC were washed twice with Roswell Park Memorial Institute 1640 medium (Bio-Whittaker, Verviers, Belgium) and suspended in the same medium, supplemented with heat-inactivated fetal bovine serum (100 ml/l; Bio-Whittaker), after decomplementation, and containing 1% penicillin–streptomycin (5000 IU/ml (3 mg/ml), 5000 mg/ml; Bio-Whittaker). The PBMC suspension was adjusted to 2×10^6 cells/ml, and 1×10^6 cells were used per well in all experiments.

Live bacterial cell suspensions of each individual *Bifidobacterium* strain or the combinations representing the faecal microbiota composition of BF and FF infants were washed in the culture medium and incubated at a final concentration of 10^7 colony-forming units/ml with PBMC (proportion bacteria:PBMC, 10:1)[15,16] during 48 h (5% CO_2 and 37°C). The supernatant was collected, centrifuged and frozen in aliquots at −80°C until cytokine analysis.

Co-culture of Caco-2/peripheral blood mononuclear cells and bacterial stimulation

The colonic adenocarcinoma cell line Caco-2 (ECACC no. 86 010 202, Salisbury, Wiltshire, UK) was cultured at 37°C and 5% CO_2 in Eagle's minimal essential medium (Bio-Whittaker) supplemented with 10% fetal bovine serum (Bio-Whittaker), 1% non-essential amino acid solution (Bio-Whittaker), 1% L-glutamine (Bio-Whittaker) and 1% penicillin–streptomycin (Bio-Whittaker). Caco-2 cells were seeded at a density of 8×10^4 cells/well in standard twenty-four-well culture plates, and at 4×10^4 cells/well on 12 mm inserts in twenty-four-well cell culture plate assemblies (Millipore, Madrid, Spain) with a semipermeable polyethylene terephthalate membrane (1 μm in pore size). During cell growth and differentiation, the medium was changed every 2 or 3 d. Once the cells were confluent and differentiated, the experiments were performed 10–11 d after seeding. Confluence was followed by microscopic visualisation and transepithelial resistance measurements (Millicell ERS Ohmmeter; Millipore).

Co-cultures of the bifidobacteria with Caco-2 cells and PBMC from healthy donors were performed in seven different experiments. To that end, a transwell cell culture system was used as described earlier. Caco-2 monolayers were challenged by apical addition of 2×10^6 colony-forming units/insert of a *Bifidobacterium* strain or a combination of strains corresponding to the species composition in the faecal samples from BF and FF infants. The PBMC suspension (500 μl) was added at a concentration of 2×10^6 cells/ml in the basal compartment of the culture well for a 12 h incubation. Thereafter, further 36 h incubation was allowed after disassembly of the system. In order to measure the cytokine production by the sensitised Caco-2 and PBMC separately, the basolateral compartment of Caco-2 cells was replenished with a fresh culture medium. After the incubation period, culture media, from both the separated PBMC and Caco-2 cell plates, were collected and frozen in aliquots at −80°C. The PBMC supernatant was centrifuged before freezing to avoid the presence of cells in aliquots.

In two different wells, two more conditions, which served as a control of the Caco-2 cell conditioning by the underlying PBMC, were carried out: the BF and FF mixtures were added to the Caco-2 monolayers in transwells with no PBMC in the basal compartment.

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Cytokine quantification in culture supernatants

TNF-α, IL-1β, IL-10, IL-8 and IL-6 cytokines were measured in the basolateral medium with Caco-2 cells, and TNF-α, interferon-γ (IFN-γ), IL-6, IL-10, IL-2 and IL-4 were measured in the PBMC supernatant. All cytokine measurements were performed using a cytometric bead array system (Inflammation Kit and either a Th1/Th2 kit or a Flex set; BD Biosciences, San Agustín de Guadalix, Madrid, Spain), according to the manufacturer's protocols, and analysed by flow cytometry (FACScalibur; BD Biosciences). Data were analysed using Cellquest software (BD Biosciences). The cytometric bead array limit of detection for each cytokine was as follows: IFN-γ, 7·1 pg/ml; TNF-α, 2·8 pg/ml; IL-10, 0·13 pg/ml; IL-6, 1·6 pg/ml; IL-8, 1·2 pg/ml; IL-4, 2·6 pg/ml; IL-2, 2·6 pg/ml; IL-1β, 7·2 pg/ml. IFN-γ was also measured with high-sensitivity Immunoassay xMAP Technology (Millipore) in a Luminex 100 equipment, with a sensitivity of 0·29 pg/ml.

Statistical analyses

Statistical analyses were performed using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). To establish the homogeneity of variances and the distribution of the data, the Levene test was run. As a result of the non-normal distribution of the data and the non-homogeneity of the variances, the Mann–Whitney *U* test was used to assess the effect of every experimental condition compared with the other conditions. Data are expressed as medians and quartiles. Significant differences were established at $P<0·05$. Correlations between different bacterial stimulatory conditions were analysed by Spearman's correlation test and considered significant at a P level $<0·05$.

Ethical approval

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects/patients were approved by the Ethics Committees of the Hospital Puerta de Hierro (Madrid, Spain) and CSIC organisation. Written informed consent was obtained from all subjects/patients.

Results

Cytokine production by peripheral blood mononuclear cells cultured with bifidobacteria

In order to determine the immunological effect of bifidobacteria on PBMC, the production of IFN-γ, TNF-α, IL-10, IL-6, IL-4 and IL-2 was measured in the supernatants of PBMC cultured in direct contact with the different *Bifidobacterium* strains (individually or mixed). Among all cytokines analysed, only IL-2 was not stimulated (Fig. 1(A)), with levels below 20 pg/ml (except for the positive control with phytohaemaglutinin; data not shown). All the other cytokines were significantly stimulated by all bifidobacterium species and their mixtures (compared with the control with only medium).

Regarding IFN-γ production (Fig. 1(B)), *B. catenulatum* and *B. breve* were the strongest enhancers, followed by the FF and BF mixtures (no significant differences were found between the two mixtures). *B. catenulatum* induced a higher IFN-γ production than all the other stimuli (except for *B. breve*). *B. breve* induced a higher IFN-γ production than *B. adolescentis*, *B. angulatum* and *B. infantis*, but similar to that induced by *B. catenulatum*, *B. longum* and the mixtures. The total percentage of *B. catenulatum* and *B. breve* was similar in the FF and BF mixture (22·05 and 25·12 %, respectively). This might explain why the levels of IFN-γ produced by PBMC stimulated with the FF and BF mixtures were not statistically different.

B. longum and *B. catenulatum* induced the highest IL-10 production by PBMC, showing significant differences in IL-10 production in the presence of *B. infantis* and the BF mixture (Fig. 1(C)). IL-10 production induced by *B. longum* was also significantly higher than that induced by *B. angulatum*, *B. breve* and the FF mixture. The percentages of *B. longum* in the FF and BF mixtures were very similar (10·87 *v.* 13·52 %), but *B. catenulatum* was approximately four times higher in the FF mixture than in the BF mixture (14·84 *v.* 3·50 %). The low proportion of *B. catenulatum* and *B. adolescentis*, together with the high proportion of *B. infantis* and *B. breve* in the BF mixture, might explain the significantly lower production of IL-10 induced by the BF mixture compared with that induced by *B. adolescentis*, *B. catenulatum* and *B. longum* individually (Fig. 1(C)). Regarding IL-4, *B. catenulatum* also induced a significantly higher production than *B. adolescentis* and *B. infantis* (Fig. 1(D)).

All *Bifidobacterium* strains stimulated PBMC to produce very high levels of IL-6, over 4000 pg/ml (Fig. 1(E)). *B. adolescentis* induced the highest IL-6 production, significantly higher than *B. angulatum*, *B. breve* and *B. infantis* ($P=0·029$ in every case). *B. infantis* induced the lowest effect among the assayed strains on cytokine production, not only for IL-6, but also for IFN-γ, IL-10 and IL-4.

With the exception of *B. adolescentis*, all *Bifidobacterium* strains also stimulated PBMC to produce very high levels of TNF-α (Fig. 1(F)). A significantly higher TNF-α production was induced by *B. angulatum* and *B. catenulatum* compared with *B. adolescentis*, *B. breve* and *B. longum*. While *B. longum* and *B. adolescentis* induced a high production of IL-10, they both mildly induced TNF-α production (Fig. 1(C) and (F)). On the other hand, while *B. infantis* and *B. angulatum* induced a mild production of IL-10, they both highly induced TNF-α production (Fig. 1(C) and (F)).

Cytokine production by peripheral blood mononuclear cells in co-culture with Caco-2 cells and bifidobacteria

To analyse cytokine production by PBMC conditioned by previous co-culture with Caco-2 cells stimulated with bifidobacteria, IFN-γ, TNF-α, IL-10, IL-6, IL-4 and IL-2 were measured in PBMC supernatants. IL-2 and IL-4 were not detectable, and TNF-α was also below the limit or approaching the limit of detection (data not shown). No significant differences in IL-10 and IL-6 production were found, either between different bifidobacteria alone or in mixtures (Fig. 2(A) and (B)).

The production of IFN-γ by PBMC was low in this system (range 1–93 pg/ml and under the detection limit in two of

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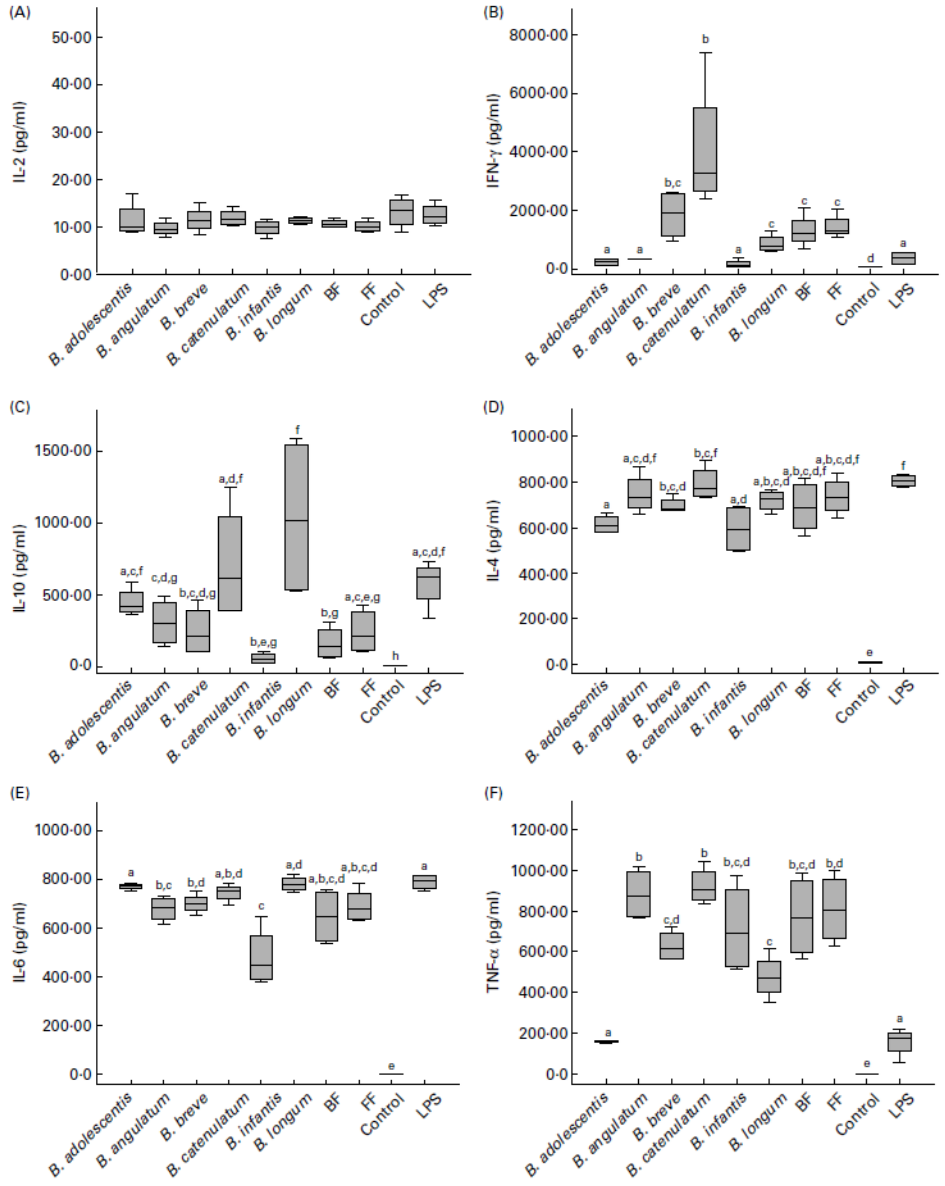

Fig. 1. Cytokine production by peripheral blood mononuclear cells after 48h incubation with individual bifidobacterium strains and their mixtures (breast-fed (BF) and formula-fed (FF)) in a 10:1 (bacteria:cell) ratio. Each box represents median (50th percentile) and interquartile range (25th and 75th percentiles). [a,b,c,d,e,f,g] Mean values with unlike letters were significantly different ($P<0·05$; Mann–Whitney *U* test). No differences were observed in IL-2 production between the conditions. (A) IL-2; (B) interferon-γ (IFN-γ); (C) IL-10; (D) IL-4; (E) IL-6; (F) TNF-α. LPS, lipopolysaccharide.

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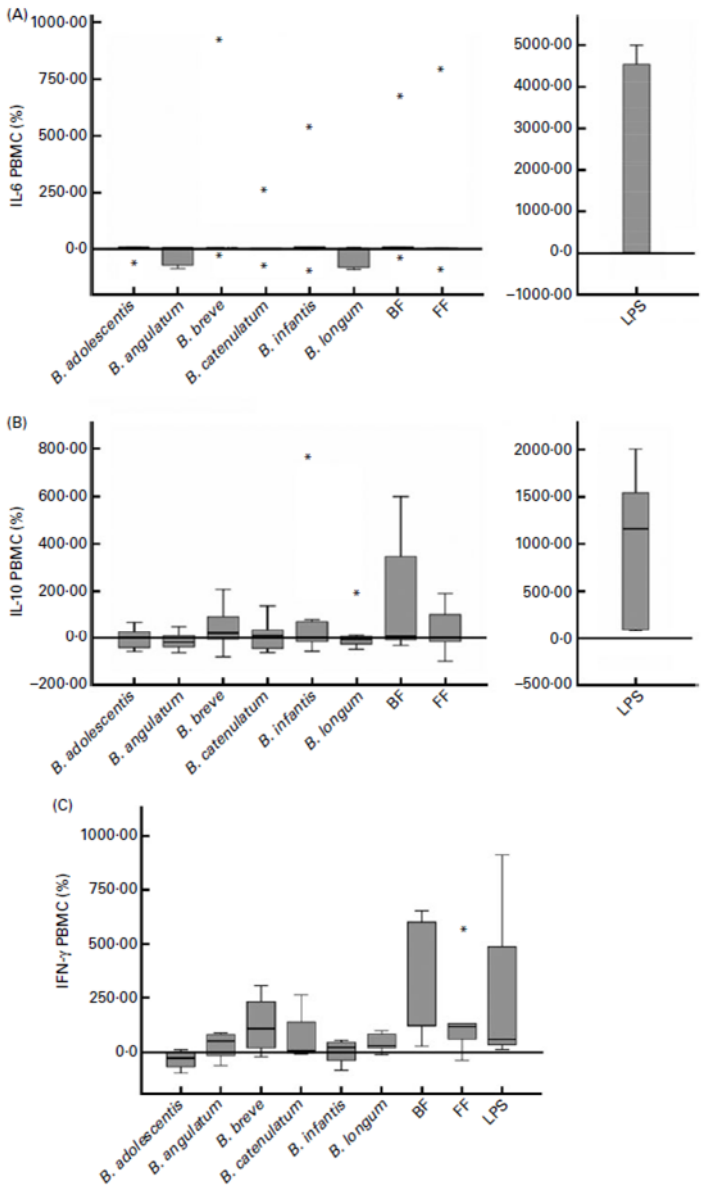

Fig. 2. Cytokine production in a 48 h culture of peripheral blood mononuclear cells (PBMC) sensitised by a 12 h incubation in a transwell co-culture system with Caco-2 cells apically stimulated with bifidobacteria. Values are given as percentage of the control (spontaneous production with no added bacteria). Each box represents median (50th percentile) and interquartile range (25th and 75th percentiles). Asterisks represent outliers. No differences were observed in IL-10 and IL-6 production between the different bifidobacterium conditions employed; however, lipopolysaccharide (LPS)-stimulated production was always significantly higher than the rest of the conditions. (A) IL-6 PBMC; (B) IL-10 PBMC; (C) interferon-γ PBMC. BF, breast fed; FF, formula fed.

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the seven PBMC donors). Using the available data from the other five donors, we found induction of IFN-γ production by the BF mixture in four of them (>100 % *v.* control) and in three of them also with *B. breve* (>50 % *v.* control), which is singularly high in the BF combination. Moreover, three donors showed stimulation with the FF mixture (>100 % *v.* control). The BF mixture was the stimulus that induced the highest IFN-γ production (Fig. 2(C)), significantly higher than *B. adolescentis* (P=0·014), *B. infantis* (P=0·050) and *B. longum* (P=0·047) individually. Although *B. breve* also induced the production of IFN-γ, this effect was not significantly different from the other bifidobacteria (Fig. 2(C)). The effect of *B. adolescentis* on IFN-γ induction was inhibitory relative to the control condition and was significantly different from the stimulatory effect observed with the bifidobacteria mixtures and *B. longum* (Fig. 2(C)).

Cytokine production by Caco-2 cells in co-culture with peripheral blood mononuclear cells and bifidobacteria

To assess the effects of bifidobacteria and bifidobacteria mixture stimulation on Caco-2 cells in co-culture with PBMC, TNF-α, IL-1β, IL-10, IL-8 and IL-6 cytokines were measured in both apical and basolateral media. All cytokines were not detectable in the apical medium, while in the basolateral medium only IL-8 and IL-6 were in a measurable concentration range (IL-8, 120–14 000 pg/ml; IL-6, 30–600 pg/ml). When Caco-2 cells were stimulated with the bifidobacteria alone, with no PBMC in the underlying compartment, the stimulation of both cytokines was three to four times lower than in the co-culture system (data not shown).

When in co-culture with PBMC, *B. breve* highly stimulated the production of IL-6 and IL-8 on Caco-2 cells (66·8 and 45·5 %, respectively; Fig. 3(A) and (B)). For IL-8, this production was significantly higher, compared with *B. adolescentis* (P=0·035), *B. infantis* (P=0·025) and the FF mixture (P=0·013) (Fig. 3(B)). Although the BF mixture also induced IL-6 and IL-8 production (36·0 and 20·7 %, respectively), these values were not significantly higher than those induced by the FF mixture (Fig. 3(A) and (B)). No significant differences were observed for IL-6 production between the different stimuli assayed (Fig. 3(A)).

Considering the PBMC donors individually, IL-8 and IL-6 production stimulated by the FF mixture was positively and significantly correlated with IL-8 and IL-6 production stimulated by *B. infantis* (P<0·001 for both cytokines). On the other hand, IL-8 production stimulated by the BF mixture was correlated with *B. angulatum*, *B. breve* and *B. catenulatum* (P<0·05), and IL-6 stimulated by the BF mixture correlated with *B. adolescentis* and *B. catenulatum* (P<0·05).

Discussion

Bifidobacterium strains have shown the capacity to modulate cytokine production by IEC, monocyte-derived dendritic cells and PBMC in *in vitro* experiments[1,8,9]. Trying to define this immunomodulatory capacity seems relevant in order to understand their contribution to the establishment of mucosal

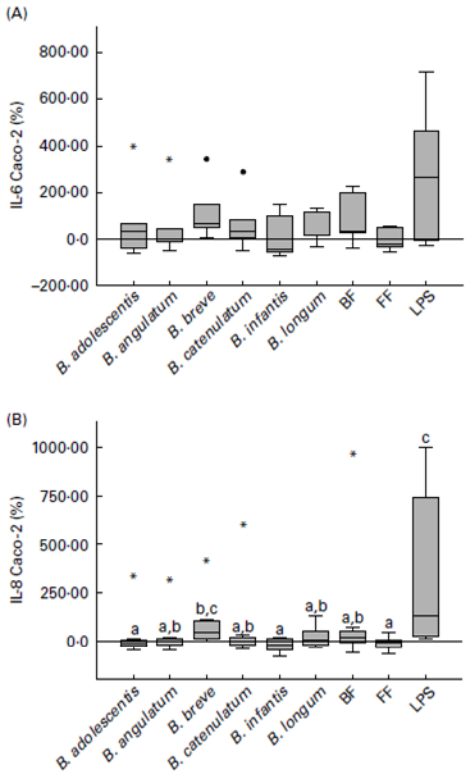

Fig. 3. Bifidobacteria-stimulated cytokine production by Caco-2 cells in a 36 h culture (basolateral medium) following prior 12 h sensitisation with peripheral blood mononuclear cells in a transwell co-culture system. Values are given as percentage of the control (spontaneous production with no added bacteria). Each box represents median (50th percentile) and interquartile range (25th and 75th percentiles). Asterisks and dots represent outliers and extreme values, respectively. [a,b,c] Mean values with unlike letters were significantly different (P<0·05; Mann–Whitney U test). No differences were observed in IL-6 production between the conditions. (A) IL-6 Caco-2; (B) IL-8 Caco-2. BF, breast fed; FF, formula fed; LPS, lipopolysaccharide.

tolerance and balanced intestinal immune responses in the early stages of life. Both these processes have been linked to the prevention of immune-mediated disorders later in life, such as allergies or inflammatory bowel disease[17,18]. Several studies have evaluated the effect of different bifidobacteria in the production of cytokines by Caco-2 cells and PBMC[6,9,19–21], but according to our knowledge, this is the first time that the *Bifidobacterium* strains used in the present study have been employed in co-culture experiments, and that the mixtures in the proportions of a FF and BF infant's typical microbiota have been used to stimulate these cell types.

In the present study, the levels of several cytokines were measured in two different systems: (1) a direct stimulation of PBMC with bifidobacteria and (2) a PBMC/Caco-2 cell

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co-culture with bifidobacteria stimulating the top layer of Caco-2 cells, which, in turn, can interact with underlying PBMC through soluble mediators. Reciprocally, PBMC were able to influence Caco-2 cell activity as well. The profile of cytokine production by PBMC exposed directly to the *Bifidobacterium* strains shows relevant differences compared with the profile of cytokine production by PBMC in the co-culture system, where Caco-2 cells constitute a physical barrier preventing the access of PBMC to the bifidobacteria. The first differential finding was that the level of cytokine production was much lower in the co-culture system. For instance, while three out of six cytokines measured were above 1000 pg/ml when both bifidobacterial mixtures were used, and two out of the remaining three gave results higher than 100 pg/ml in direct contact, only IL-6 by PBMC in the co-culture system gave results higher than 1000 pg/ml. It is worth noting that while in direct contact, IL-6 and TNF-α were the cytokines most highly induced, in the co-culture system, not only IL-6 but also IL-10 was the cytokine most highly produced by PBMC. In this sense, Niers *et al.* showed in a single culture system that the production of IL-10 by PBMC is boosted by several *Bifidobacterium* strains, and this down-regulates the production of TNF-α and IL-12p70 by these cells. When they used a monoclonal antibody against IL-10, they found a huge increase in the production of these inflammatory cytokines.

Different cytokines (IL-8 and IL-6) were also stimulated on Caco-2 cells, but only when they were previously co-cultured with PBMC; no cytokine production was measured if the Caco-2 cells were cultured alone with the *Bifidobacterium* strains. Therefore, the presence of PBMC is an essential factor for the sensitisation of Caco-2 cells to respond to bifidobacteria, which is presumably exerted by the communication between the two cell types through soluble mediators. In the present study and other studies, Caco-2 cells alone have been found to be hyporesponsive to bifidobacteria stimulation[22] and also to other probiotic bacteria[21,22]. Moreover, since cytokine production by Caco-2 cells in the co-culture system was only detectable in the basolateral medium and not in the apical medium, it demonstrates a polarised secretion by Caco-2 cells, as have been found earlier by other authors[21]. In a similar co-culture system, in which Caco-2 cells were stimulated with non-pathogenic *Escherichia coli* and *Lactobacillus sakei*, an induction of TNF-α secretion into the subepithelial compartment was observed, and this cytokine was signalled as the fundamental candidate for cellular crosstalk[21]. In contrast, we found no detectable production of TNF-α, which might be explained by a differential effect from different bacterial species and strains.

Regarding the immunomodulatory effects of specific strains used in these experiments, the most relevant findings have been found regarding the immunostimulatory effects of *B. breve*. This strain stimulated most of the production of IL-8 and IL-6 on both Caco-2 cells and PBMC. In the microbiota of BF infants, *B. breve* is the most representative *Bifidobacterium* species (after *B. infantis*, common in all milk-fed babies), and this could explain the high IL-8 and IL-6 levels produced by Caco-2 and PBMC stimulated with the BF mixture. This link between *B. breve* and the BF mixture was supported by the correlation found between IL-8 levels produced by Caco-2 cells stimulated by *B. breve* and the BF mixture. Moreover, *B. breve* and the BF mixture also stimulated the production of IL-10 and INF-γ by PBMC (in co-culture with Caco-2 cells). All these observations might indicate that the proportion of different *Bifidobacterium* species is an important determinant of the overall contribution to the stimulation of cytokines on the intestinal mucosa. In this sense, it is interesting to note that there was a correlation between the relative inhibition of IL-8 production by Caco-2 cells induced by the FF mixture and *B. infantis*. It seems that the differences in the proportions of the different strains between the mixtures and the stimulatory/inhibitory capacities shown by the individual strains might explain the results found with their combinations in the BF and FF mixtures.

According to the results, *B. breve* induced a slight pro-inflammatory response, which could turn the mucosal immune system on stand-by and prevent the release of a severe inflammation. It has already been reported that infants from 4 to 6 months old, who daily consumed infant formula fermented with *B. breve* and *Streptococcus thermophilus*, presented less severe episodes of acute diarrhoea than the standard formula group[23]. Furthermore, Li *et al.*[24] showed that the administration of *B. breve* to low-birth-weight infants was useful in promoting the colonisation by other bifidobacteria, which might contribute to the establishment of a healthier microbiota. More recently, it has been found that the administration of *B. breve* to pre-term infants can up-regulate transforming growth factor-β1 signalling and may possibly be beneficial in attenuating inflammatory and allergic reactions in these infants[25].

In allergic models, some probiotic bifidobacteria have the capacity to suppress IL-4 production, *in vitro*[16] and *in vivo*[26]. We have observed that not all bifidobacterial species induce the same IL-4 production (Fig. 1(D)), indicating different effects of the interaction between bifidobacteria and PBMC related to the species.

Regarding the stimulation of the regulatory cytokine IL-10 by PBMC after direct stimulation with *B. longum*, a similar finding has been previously described by Medina *et al.*, who found that several strains of *B. longum* are strong inducers of IL-10 secretion on PBMC. On the other hand, the finding that *B. infantis* is a weak inducer of cytokine secretion after direct stimulation of both PBMC and Caco-2 cells is in agreement with prior published results that have described that *B. infantis* attenuates baseline IL-8 secretion in HT-29 epithelial cells[5] as well as pro-inflammatory IL-17 production by murine splenocytes and dextran sodium sulphate-induced intestinal inflammation[27,28].

In conclusion, among the *Bifidobacterium* species tested, *B. breve* seems to be the most immunostimulatory strain in a co-culture system resembling the physiological layout of different cell types in the intestinal mucosa. The presence and relative proportions of different *Bifidobacterium* species in the microbiota of BF and FF infants could be key factors defining the immunomodulatory effect of the gut microbiota in early life.

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